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THE DISTRIBUTION OF POLYSACCHARIDES AND BASOPHILIC SUBSTANCES DURING THE DEVELOPMENT OF THE MUSHROOM COPRINUS[1]

JOHN TYLER BONNER, ALLAN A. HOFFMAN, WILFRED T. MORIOKA AND A. DUNCAN CHIQUOINE

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As Buller (1909 *et seq.*) has pointed out, some species of the genus *Coprinus* sow their spores once during a short interval of time and the fruiting body disappears shortly thereafter by auto-digestion. Characteristically the small buds will, all in one day, go through a period of rapid expansion and elongation, shed their spores, and deliquesce. This rapidity is no doubt related to their small size, for larger species of hymenomycetes will go through many days of continued production and shedding of spores.

The origin of the gills and their individual lamellae has been described in detail by Atkinson (1916). They are formed at an early stage by the orientation of hyphae and the final result (which is illustrated in Figure 1) has a number of distinguishable component parts: the outside is covered with the hymenium which consists of a mixture of basidia and sterile paraphyses; below this there is a subhymenial layer of small hyphae; and finally the central portion of the gill lamella, the tramal layer, which is composed of large hyphae.

Borriss (1934) has shown that the development of *Coprinus* occurs in two distinct stages, one in which cell division and the initial cell orientation takes place, and it is during this stage that the gill primordia are formed. The second stage consists of rapid cell elongation and in the latter phase of this period the spores bud from the basidia. It may be inferred, from the recent results of Madelin (1956), that this period of rapid expansion involves the transfer of material from the vegetative mycelia. Such a transfer is in keeping with the views of Buller as well as with the situation in *Agaricus campestris* (Bonner, Kane and Levey, 1956).

Using histochemical techniques, it has been possible in this study to follow the distribution of certain groups of substances in the fruiting body. It could be demonstrated that these substances accumulated at specific locations in the gills and that they were all transferred into the spores, so that by the advent of auto-digestion there were virtually no demonstrable substances left within the cells at the time of the final destruction. Not only does this indicate an efficiency, an economy in the

[1] This research was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

fruiting process, but also it is of some interest to find that prior to sporulation the different substances are stored in different parts of the gills.

Methods

Two species of *Coprinus* were studied: *C. lagopus* Fr. and *C. curtus* Kalch. (The authors would like to thank Dr. Haig P. Papazian of Yale University for the culture of *C. lagopus*, and Dr. Alexander H. Smith of the University of Michigan for the identification of *C. curtus*.) They were grown in jars on moist, sterile horse dung and kept at room temperature (approximately 24° C.)

The entire fruiting bodies were fixed for 12 hours in Rossman's fluid (9 parts absolute alcohol saturated with picric acid plus 1 part formaldehyde) at 4° C. The selection of this particular fixative, as well as the temperature, follows from the recommendations of Deane *et al.* (1946), from their studies on the preservation and localization of glycogen in mammalian liver. The suitability of the fixation for the mushroom material is evidenced by the absence of any false localization of glycogen, "glycogen flight," due to the pathways of penetration of the fixative. Following fixation the tissues were dehydrated, cleared with methyl salicylate (14 hours), and embedded in paraffin. Sections were cut at both 5 and 10 μ and mounted on slides in the usual manner.

Polysaccharides were demonstrated by the periodic acid-Schiff reaction as described by Gomori (1952). This technique is based upon the oxidation with periodic acid of the vicinal hydroxyl groups of polysaccharides to aldehyde groups and the subsequent visualization of the aldehyde groups by reaction with the Schiff reagent. The sections were deparaffinized, run to water, and oxidized for 10 minutes at room temperature with 0.5% periodic acid. Schiff reagent was prepared according to the method of Lillie (1954) and the slides were treated with it for 20 minutes. Excess Schiff reagent was removed by three sodium metabisulphite rinses, the slides dehydrated, cleared and mounted. Control sections were treated exactly the same, although not oxidized with periodic acid. Any material which stained with the PAS technique and did not stain in the control slides is hereafter referred to as polysaccharide.

It was possible to differentiate mucopolysaccharides from glycogen by digestion of the slides with salivary amylase prior to the periodic acid oxidation. The slides were run to water as before and digested for three hours at room temperature in saliva. Following digestion the slides were run through the same PAS procedure outlined above. Any material which was PAS-positive but removable by salivary digestion is hereafter referred to as glycogen. Any material not removable with salivary digestion is referred to as mucopolysaccharides. Additional slides placed in water for three hours, in place of saliva, served as control slides. Since the pattern of localization in those control slides differed in no way from the standard PAS pattern, one can conclude that the saliva removes glycogen by enzymatic hydrolysis specific for glycogen and not by a leaching-out of materials by simple dissolution in an aqueous solution.

Regions of basophilia were demonstrated in the same material by staining with a 0.1% aqueous solution of toluidin blue. Cytoplasmic basophilia can be attributed to the presence of ribose nucleoproteins and/or acid mucopolysaccharides. Since all

basophilic material observed in this study was not PAS-positive, it is assumed that the basophilia was due to ribose nucleoproteins.

Results

A series of stages of both species of *Coprinus* was fixed and sectioned. Then duplicate slides of each fruiting body were stained by (1) the PAS method for all polysaccharides, (2) the PAS method with salivary digestion for the mucopoly-

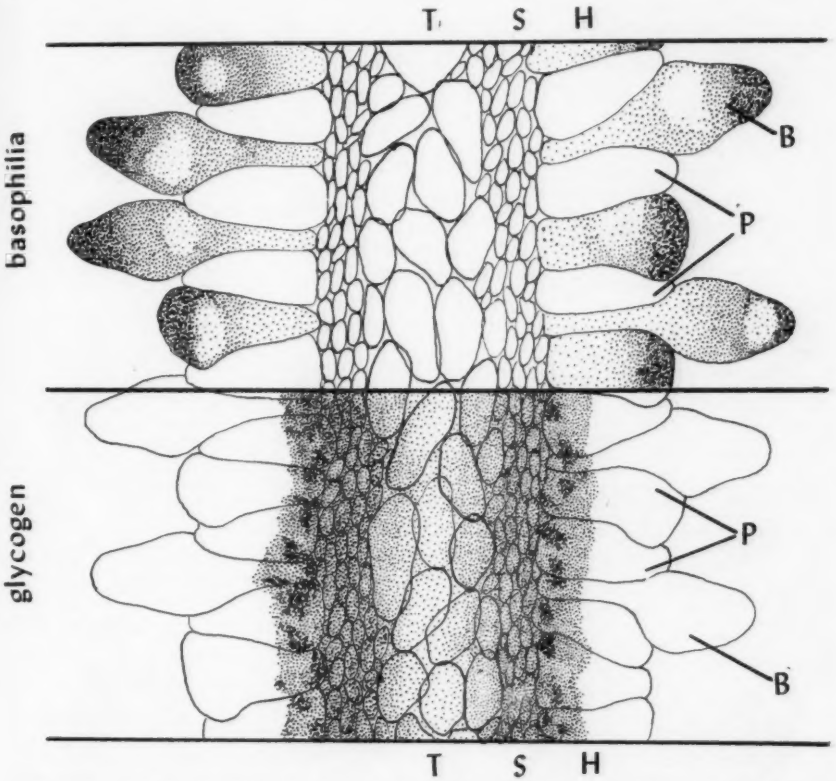

Figure 1. A section through a gill indicating the distribution of basophilic substances (above) and glycogen (below). T, tramal layer; S, sub-hymenium; H, hymenium; B, basidium; P, paraphysis. (Drawing by Miss Marcia J. Shaw.)

saccharides, and (3) with toluidin blue for the basophilic substances. Therefore, it was possible to compare a specific fruiting body at a specific stage for three substances: mucopolysaccharides, glycogen, and basophilic substances.

First of all it should be said that both species, *C. lagopus* and *C. curtus*, showed the same staining characteristics, and therefore the description given below applies

to both. The general staining properties of the cap and stipe could be seen more advantageously in the smaller *C. curtus*, while *C. lagopus* was especially suitable for examination of the gills.

An intense staining with Schiff reagent was observed in the globular cells of the cap in *C. curtus* which was not dependent upon periodic acid oxidation. There was also a faint staining of all cell walls in non-oxidized control sections, and character-

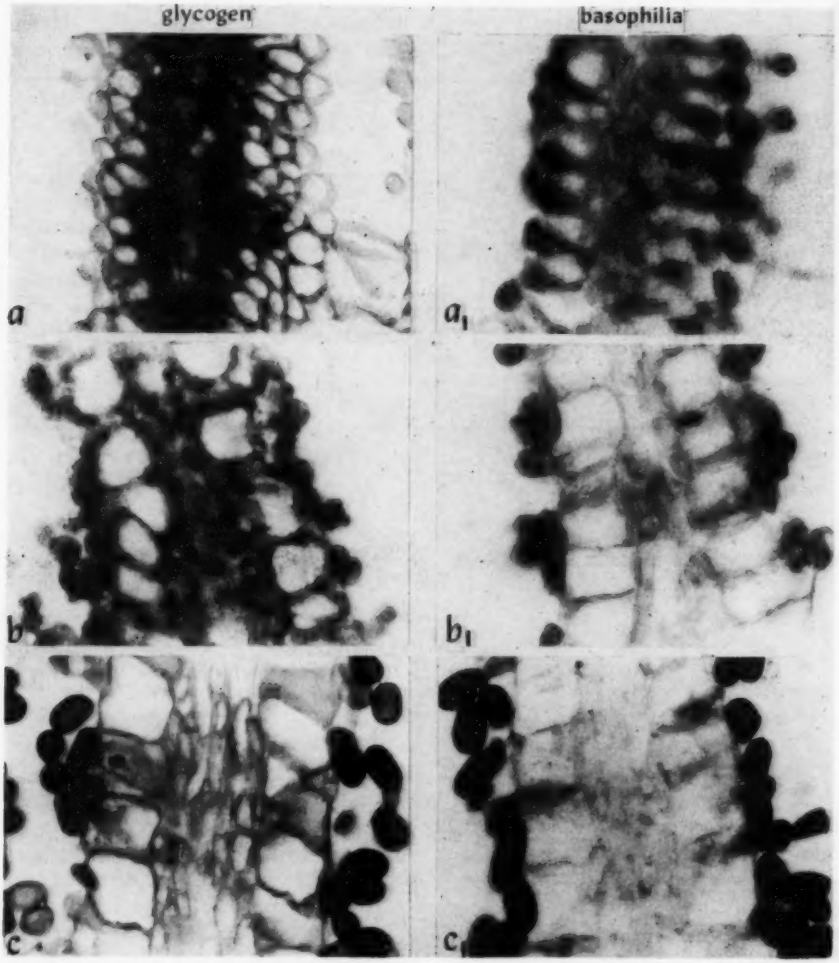

FIGURE 2. Photograph of sections through the gills showing the distribution of glycogen (left column) and basophilic substances (right column) at different stages of development; a, b, c represent the three stages of before, during, and after spore formation. Each pair (*e.g.*, a and a_1) are from the same mushroom.

istically the stain was more intense at the basal end of the mushroom. The basis for these two staining reactions is unknown. Following periodic acid oxidation the cell walls stained intensely, indicating the presence of mucopolysaccharides, presumably chitin. The cell walls were the only regions where mucopolysaccharides were observed in a significant concentration.

Exclusive of the gills, there was no general difference in the distribution of glycogen and the basophilic substances; they both appeared to be present in small quantities all over the mushroom at early stages, although the growth zone just below the cap always showed a higher concentration. The intensity of staining reactions was inversely proportional to the cell length, that is, the short cells of the growth zone at early stages were darkly stained, but after complete expansion these cells were depleted of stainable material as were the cells of the rest of the mushroom.

In the gills, prior to spore formation, there is a dark layer showing a concentration of basophilic substances, as well as a similar layer of glycogen. The interesting point is that, as shown in Figures 1 and 2, the positions of these layers do not correspond. The basophilic layer lies at the tips of the basidia, that is, the end at which the future spores will be formed. Furthermore, basophilia is observed only in the basidia and not in the paraphyses. The glycogen lies primarily in the subhymenium, although it extends to the base of the hymenium (in both the basidia and paraphyses) on one side and into the tramal layer on the other.

This staining picture is present at the earliest stages that gills are discernible, and in subsequent stages there is no obvious increase in the intensity of the staining. The only significant change is the increase in the size and the extent of the gills with age.

It was possible to show that once the spores are formed (Fig. 2, c) both the stainable layers disappear, showing a complete absence of glycogen and basophilic substances. Therefore, it is presumed that these materials enter into the spores, but the spores themselves become darkly pigmented with maturity, which masks any staining reaction they might show.

In order to verify the hypothesis that the spores contain these substances, a new series of mushrooms of intermediate size was fixed to find ones in the middle of the process of spore formation. A particular set of sections of *C. lagopus* was fortunately exactly at this stage (Fig. 2, b). The glycogen is now present in and around the hymenium, as well as concentrated in the young spores. The basophilic substances, which were at the basidial tips to begin with, appear to enter directly into the spores during their formation.

Discussion

The presence of glycogen about the base of the basidia, and its outward movement during spore formation, suggest that it might supply energy for the process of spore formation. However, a considerable portion of the glycogen enters directly into the spore and becomes part of its reserves for future germination and growth.

The basophilic substances are at the end of the basidium that forms the spores and therefore the material is transferred directly upon spore formation. Presumably the nuclear material is associated with this basophilic zone and both are transferred together.

It is of interest to note that the staining appears equally intense in the gills in

both small young mushrooms and large fully developed ones. The only difference is the extent of the gill material and from this we might presume that the gill is laid down with all its food material for sporulation right in the beginning, and during the period of growth the sole change is that the total amount of gill material is extended. From the work of Madelin (1956) it is likely that materials are constantly being drawn up from the vegetative hyphae into the mushroom during its expansion, and this material must be led directly to the newly forming gill.

The observation that essentially all stainable glycogen and basophilic substances are gone after sporulation implies an economy, and one would expect this phenomenon to be correlated with the fact that in small species of *Coprinus* there is but one short period of spore formation and discharge. Mr. Anthony J. Schmidt of this laboratory has made some preliminary PAS and toluidin blue preparations of *Agaricus campestris* and here one finds large concentrations of glycogen and basophilic substances in the cap with channels of conduction to the hymenium. Therefore in a large species which forms spores over a long period there is a large store of substances that can be continuously poured into the spores.

Summary

Two species of *Coprinus* (*C. lagopus* and *C. curtus*) were examined, using histochemical techniques, and it was found that prior to sporulation there were two distinct zones in the gills, one containing glycogen and one containing basophilic substances. The glycogen zone is at the base of the hymenium, extending into the central tramal layer. The basophilic zone is at the outer tips of the basidia. Upon sporulation both these groups of substances entered the spores, leaving no demonstrable material within the cells of the gills.

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RELATION BETWEEN POSITION OF BURROWS AND TIDAL RHYTHM OF UCA[1]

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The literature concerning tidal and semilunar rhythms of color change has been reviewed by Fingerman (1957). These rhythms were first observed in the fiddler crab *Uca pugnax* collected in the region of Woods Hole, Massachusetts, where the tides are semidiurnal (Brown, Fingerman, Sandeen and Webb, 1953). The crabs darkened by day and lightened by night in accordance with their 24-hour rhythm of color change. Superimposed upon the latter was a tidal rhythm that progressed across the 24-hour rhythm at the average rate of 48.8 minutes per day, as evidenced by a supplementary dispersion of pigment in the melanophores about the time of low tide. Also evident was a semilunar rhythm of 14.8-day frequency, the average interval between days on which the 24-hour and tidal rhythms repeat similar time relations to one another. The relationship between the time of supplementary dispersion of the melanin and the time of low tide was determined directly by the local tidal situation where the crabs were collected, as evidenced by the phase difference of the tidal rhythms of *Uca pugnax* collected from two localities where the times of low tide were different.

Persistent tidal and semilunar rhythms of color change have been observed in the blue crab, *Callinectes sapidus*, by Fingerman (1955). The rhythms were similar to those described above for *Uca pugnax* in spite of the fact that the *Callinectes* were collected in the vicinity of New Orleans, Louisiana, a region of diurnal tides. Evidently, the center of tidal rhythmicity in *Callinectes* operates on the basis of tides spaced 12.4 hours apart in spite of the fact that the crabs were collected in a region where 24.8 hours is the interval between two successive low tides.

Tidal rhythms of color change have been observed in two species of fiddler crabs in addition to *Uca pugnax*, *Uca pugilator* and *Uca speciosa* by Fingerman (1956). The latter two species were collected at Ocean Springs, Mississippi, where the tides are diurnal. The tidal rhythms of both species were similar in nature to the tidal rhythms of *Uca pugnax* and *Callinectes sapidus*. Both the *Uca pugilator* and the *Uca speciosa* were collected from limited portions of the beach. Analysis of the tidal rhythm of both species revealed that the *Uca speciosa* behaved as if low tide occurred for them 7.5 hours earlier in the day than low tide for the *Uca pugilator*.

Inspection of the beach at Ocean Springs revealed that the burrows of the *Uca speciosa* were closer to the high tide mark than were the burrows of the *Uca pugilator*. The phase difference of the tidal rhythms appeared to be due to the fact that when the water began to recede after high tide, a local low tide occurred earlier for the *Uca speciosa* than for the *Uca pugilator*. The *Uca speciosa* would be free, therefore, to leave their burrows and feed earlier than the *Uca pugilator* living

[1] This investigation was supported by Grant No. B-838 from the National Institutes of Health.

closer to the actual low tide mark. Measurements of the beach where both species were collected revealed that the water actually began to uncover the burrows of the *Uca pugilator* 4.9 hours after the burrows of the *Uca speciosa* began to be uncovered.

The present study was undertaken to investigate further the nature of the tidal rhythm of color change of the fiddler crab *Uca pugilator* and to test the hypothesis presented above that the phases of the tidal rhythm are set by the time of low tide at a limited portion of beach. The hypothesis was tested by comparing the phases of the tidal rhythm of color change of specimens of the same species collected from distinct sets of burrows different distances from the high tide mark, because the possibility existed that the phase difference between the *Uca speciosa* and *Uca pugilator* was a species difference and not due to the tides as hypothesized above.

Materials and Methods

Adult male and female specimens of the fiddler crab *Uca pugilator* were collected on the beach near the Gulf Coast Research Laboratory, Ocean Springs, Mississippi, for use in the observations reported below. The specific collection site consisted of two discrete sets of burrows. Crabs could be collected from each set of burrows with assurance that no mingling of the two groups occurred normally. Figure 1 is a diagrammatic representation of the section of the beach where the animals were collected. Included in this figure are the distances of the burrows from each other and from the high and low tide marks. A sub-surface drainage pipe, not shown on the diagram, emptied its contents between the two sets of burrows. The water flowing from this pipe at times of low tide was another factor that kept the crabs of the two sets of burrows isolated from one another.

The animals were placed into stainless steel aquaria and transported to an air-conditioned laboratory in New Orleans. From the evening of the day of collection until the end of the period of observation, the animals were maintained in darkness except for the few minutes required to make observations of the chromatophores and to change the water in the aquaria on days observations were performed. The animals were kept in inclined stainless steel aquaria containing sufficient sea water to cover approximately one-half of the bottom of the aquaria. The crabs were, therefore, free to move into and out of the water.

Chromatophores were staged in the manner described by Fingerman (1956). The average index was determined for the melanophores on the anterior aspect of a walking leg with the aid of a stereoscopic dissecting microscope and lamp. The chromatophore indices of Hogben and Slome (1931) were used in staging the chromatophores. The most concentrated state of the pigment is referred to as stage 1, the most dispersed stage 5, and the intermediate conditions stages 2, 3, and 4.

Results and Discussion

The measurements of the portion of the beach at Ocean Springs, where the animals were collected (Fig. 1), were analyzed to determine the phase difference that would be expected between the tidal rhythms of the animals from the two sets of burrows if the hypothesis of Fingerman (1956) is correct. The distance from the high tide mark to the low tide mark was 215 feet (Fig. 1). Twelve and four-tenths hours is the average interval required for the water to recede from the high

tide mark to the low tide mark. One set of burrows was 28 feet closer to the high tide mark than was the second set. If the water recedes 215 feet in 12.4 hours, then the water will recede 28 feet in 1.6 hours assuming, of course, that the slope is uniform. The set of burrows closer to the high tide mark would, therefore, begin to be uncovered by the receding water 1.6 hours earlier each day than would the set of burrows closer to the low tide mark. On the basis of the average tidal progression of 48.8 minutes per day, 1.6 hours in a tidal cycle are equivalent to two days. Therefore, a phase difference of two days was anticipated between the specimens from the two sets of burrows when they were examined in the laboratory.

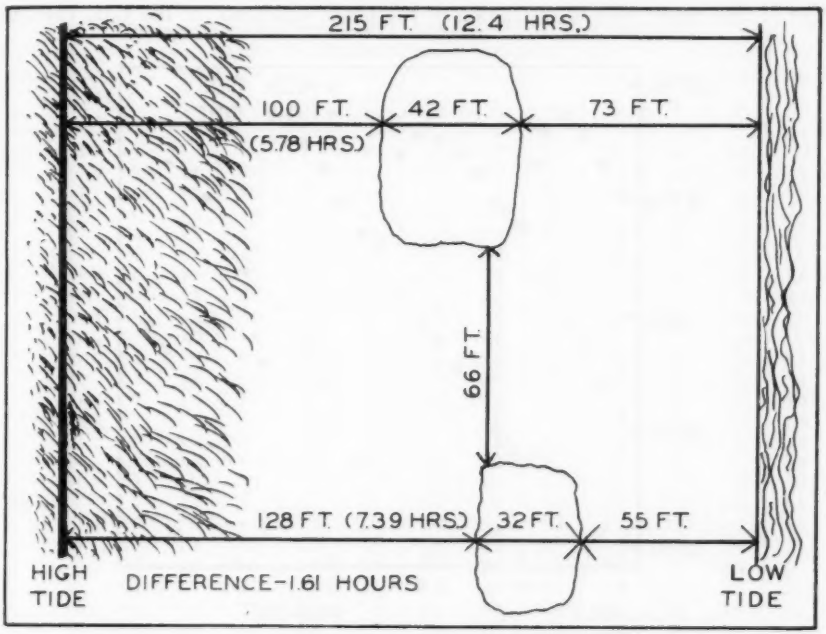

FIGURE 1. Diagrammatic representation of the positions of the burrows on the beach at Ocean Springs, Mississippi, where the specimens of *Uca pugilator* were collected.

Figure 2 presents the times of high and low tide at Ocean Springs on days chromatophores were observed in the laboratory. The diurnal nature of the tides is obvious. However, the tides did not follow the usual pattern of advancing 48.8 minutes daily across the 24-hour day. High tides occurred generally between midnight and 2 P.M. and low tides from 2 P.M. to midnight. The data for Figure 2 were selected from the tide tables published by the Coast and Geodetic Survey, United States Department of Commerce.

On July 3, 1956, several hundred crabs were collected from each set of burrows for the first series of observations that started at 8 A.M. on July 4. On days when observations were performed, 50 specimens from each group were observed hourly

from 8 A.M. through 7 P.M. and the average chromatophore stage was calculated for each group. The crabs from the burrows closer to the high tide mark will be referred to as Series A and the crabs from lower on the beach as Series B. In Series A a sufficient number of crabs survived throughout the period of observation so that 50 specimens were always available. Many of the Series B crabs died suddenly between August 6 and 8 so that this group had to be discarded. The average hourly chromatophore stages for Series A and B on each day observations were performed are presented in Tables I and II, respectively. Observations were usually made every second day, but from July 12 through 17 observations were daily.

Some of the data were taken from Tables I and II in order to prepare Figure 3. Use of this figure aids in discussion of the phase difference of the tidal rhythms

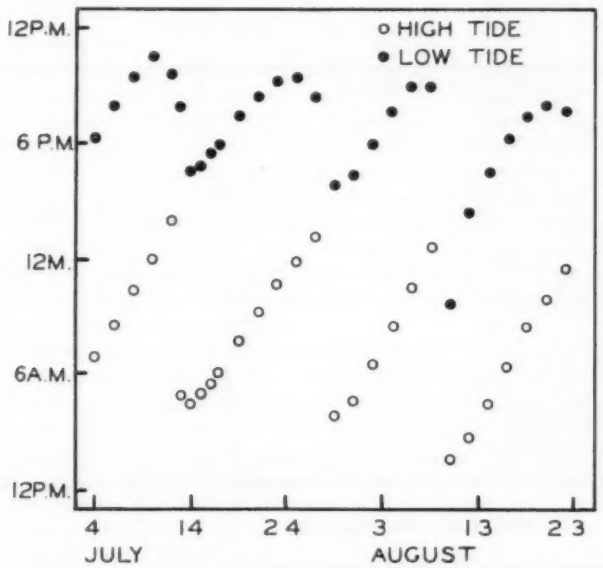

FIGURE 2. Times of high and low tide at Ocean Springs on days observations of the crabs were made in the laboratory.

of the two sets of animals. In Figure 3 the daily patterns of Series A and B crabs were arranged according to the expected two-day phase difference. According to the calculations low tide at any given hour of the day, *e.g.*, noon, would occur two days earlier for Series B crabs than for Series A crabs. Therefore, the curve of Series A for any given day was placed beside the curve of Series B that had been obtained two days previously. As is evident from inspection of Figure 3, a two-day difference in the tidal rhythms was present as shown by the similarity of the curves of Series A with the curves of Series B that had been obtained two days earlier.

The shapes of the daily curves obtained during the first three weeks of observa-

tion did not turn out as predicted on the basis of the observations of chromatophores of *Uca pugilator* performed during the summer of 1955 (Fingerman, 1956). The maxima of the curves did not progress across the day but were restricted to the left side of the curves, decidedly different from the results obtained with the same species collected in 1955 in the same area (Fingerman, 1956). The specimens of *Uca pugilator* observed during the summer of 1955 exhibited the typical pattern of tidal rhythm of color change just as was found in *Uca pugnax* by Brown, Fingerman, Sandeen and Webb (1953) and in *Callinectes sapidus* by Fingerman (1955).

TABLE I

The average melanophore index for each of the 12 daily observations of the Uca pugilator animals of Series A

		8 A.M.	9	10	11	12 M.	1 P.M.	2	3	4	5	6	7
July	4	3.7	3.4	3.6	3.3	3.6	3.8	3.8	3.4	3.2	3.6	3.3	2.9
	6	3.9	3.8	3.6	3.7	3.3	3.5	3.3	3.1	3.3	3.2	2.8	2.9
	8	3.8	4.2	4.2	3.7	3.6	3.7	3.5	3.7	4.0	3.8	3.4	3.4
	10	4.0	3.9	3.9	4.2	4.1	4.1	3.7	3.5	3.3	3.8	3.9	3.6
	12	3.3	3.2	3.9	3.9	3.6	4.0	3.8	3.2	3.5	3.4	3.2	3.1
	13	3.5	3.8	3.5	3.6	3.6	3.6	3.4	3.3	3.6	3.6	3.9	3.4
	14	3.6	3.6	3.7	3.6	3.6	3.5	3.5	3.6	3.7	3.4	3.4	3.6
	15	3.9	3.7	3.1	3.1	3.4	3.3	3.2	3.4	3.3	3.5	3.3	3.5
	16	3.8	4.0	3.6	3.4	3.5	3.2	3.0	3.1	3.1	3.4	3.1	3.2
	17	3.6	3.9	3.9	3.8	3.8	3.5	3.5	3.0	3.4	3.2	3.2	3.3
	19	3.6	3.2	3.6	3.3	3.3	3.4	3.5	3.2	3.0	3.1	2.9	2.5
	21	4.2	3.8	4.0	3.6	3.5	3.7	3.9	3.7	3.7	3.6	3.6	3.1
	23	3.9	3.8	4.1	3.2	3.4	3.0	3.3	3.2	3.3	3.2	3.0	3.6
	25	4.2	3.9	3.7	3.4	3.0	3.2	3.9	3.4	3.0	3.1	3.3	2.9
	27	3.6	3.7	3.0	2.8	3.0	3.1	3.2	3.3	3.3	3.2	3.3	2.9
	29	3.8	3.2	3.4	2.9	2.8	3.0	3.3	3.2	3.5	3.3	3.2	2.8
	31	3.6	3.9	3.2	3.0	3.1	3.3	3.0	3.0	3.0	3.3	2.9	2.8
August	2	3.6	3.4	3.5	3.2	3.1	2.9	3.2	3.0	3.1	3.2	2.9	2.7
	4	3.5	3.3	3.0	2.8	2.9	3.0	3.2	3.3	3.1	3.5	3.2	2.9
	6	2.6	3.2	3.6	2.9	3.3	3.2	3.3	3.3	3.4	3.1	2.9	3.1
	8	3.1	2.8	3.1	3.0	2.9	3.3	3.0	3.4	3.2	3.2	3.5	3.3
	10	3.0	2.9	2.7	2.6	2.8	3.1	3.0	2.8	3.2	3.3	3.5	3.0
	12	3.4	3.4	2.8	3.0	2.9	2.7	3.4	3.2	3.2	3.0	3.0	2.9
	14	3.0	3.3	3.5	2.8	3.1	2.8	2.9	3.0	2.7	2.9	2.7	2.4
	16	3.1	3.0	3.0	2.8	3.2	3.2	3.2	3.5	3.5	3.0	3.4	3.0
	18	3.3	3.1	2.7	3.2	2.8	3.1	2.8	3.1	3.0	3.3	3.0	3.1
	20	3.2	3.2	3.1	3.2	3.0	2.9	3.1	3.0	3.2	3.4	3.5	3.1
	22	3.0	3.0	2.7	2.8	3.0	2.8	2.9	2.9	3.0	3.1	3.2	3.2

The tidal maximum of pigment dispersion of the crabs observed in 1955 progressed across the 24-hour day at the usual tidal rate of 48.8 minutes per day, with the result that maximum pigment dispersion occurred in the right half of approximately 50 per cent of the curves depicting the daily pattern of pigment dispersion. However, the maximum did not progress into the afternoon during the first three weeks of observation in 1956. More will be said of this phenomenon below.

In order to reaffirm these observations specimens of *Uca* were collected on July 18, 1956, from the same sets of burrows as were the animals of Series A and B and

were treated in the same fashion as the animals collected previously. Crabs collected on July 18 from burrows nearer the high tide mark will be referred to as Series C and crabs from burrows lower on the beach as Series D. The average chromatophore indices determined for Series C and D are presented in Tables III and IV, respectively. Fifty specimens were available from Series C throughout the observations. However, with Series D 50 were available only from July 19 through August 8. The number then gradually diminished from 49 on August 10 to 26 on August 22, the last day of observation. A two-day phase difference in the tidal rhythms of Series C and D crabs was evident during the first week of observation. Maxima were restricted to the left half of the curves during this period as were the maxima of the curves of Series A and B. About July 26, however, unexpected results that will be described below appeared.

TABLE II

The average melanophore index for each of the 12 daily observations of the Uca pugilator specimens of Series B

		8 A.M.	9	10	11	12 M.	1 P.M.	2	3	4	5	6	7
July	4	3.8	3.8	3.6	3.4	3.3	3.5	3.5	3.3	3.3	3.1	2.5	2.6
	6	3.7	3.8	4.0	3.6	3.2	3.5	2.9	3.2	3.4	3.3	3.3	2.6
	8	3.6	3.8	4.1	3.9	3.9	3.6	3.7	3.8	3.2	3.6	3.6	3.2
	10	3.3	3.7	3.4	3.8	4.1	4.0	3.8	3.7	3.2	2.8	3.5	3.1
	12	3.4	3.4	3.8	3.2	3.5	3.3	3.3	3.6	3.5	3.1	3.3	3.2
	13	3.2	4.0	3.5	3.3	3.4	3.5	3.3	3.8	3.3	2.8	3.2	3.2
	14	4.0	3.7	3.7	3.3	3.2	3.5	3.0	3.3	3.3	3.3	3.2	3.1
	15	3.9	4.0	3.9	3.7	3.8	3.2	3.4	3.0	3.5	3.3	3.3	3.2
	16	3.4	3.6	3.6	3.4	4.0	3.7	3.2	3.6	3.1	3.4	3.0	3.4
	17	3.9	4.0	3.7	3.9	3.5	3.3	3.5	3.2	3.3	2.9	3.1	2.7
	19	3.4	3.9	3.7	3.5	3.4	3.3	3.3	2.9	3.2	3.1	2.8	2.4
	21	3.5	3.7	3.8	3.1	3.3	3.0	3.1	2.8	2.7	2.5	2.7	2.5
	23	3.4	3.5	3.5	3.0	2.7	2.9	2.8	3.1	2.8	2.8	2.7	2.3
	25	3.9	3.6	3.3	3.1	3.2	3.1	3.0	2.9	2.9	2.9	2.7	2.4
	27	3.2	3.2	2.7	2.8	2.5	2.6	2.7	2.6	2.5	2.5	2.6	2.3
	29	3.1	3.1	2.5	2.4	2.5	2.6	2.3	2.6	2.6	2.4	2.5	2.1
	31	3.7	2.9	2.8	2.7	2.6	2.6	2.4	2.5	2.6	2.7	2.4	2.2
August	2	3.0	3.0	2.6	2.7	2.7	2.5	2.4	2.4	2.6	2.2	2.6	2.1
	4	3.0	2.9	2.9	2.4	2.5	2.4	2.4	2.5	2.4	2.3	2.4	2.3
	6	3.2	3.0	2.9	3.0	2.8	2.8	3.1	2.8	2.9	2.6	2.3	2.2

To determine the precise rate at which the tidal rhythm progressed across the 24-hour rhythm, and to demonstrate clearly the phase difference between the tidal rhythms of the crabs from the two sets of burrows, Figures 4 and 5 were prepared as follows. The data of Series A and B were used for Figure 4 and Series C and D for Figure 5. The 12 periods of observation were divided into four periods of three hours each, 8–10 A.M., 11 A.M.–1 P.M., 2–4 P.M., and 5–7 P.M. The 12 hourly averages were summed and 12 subtracted from the total because if there had been no daily excursion of the pigment the sum would have been 12. Likewise, the average chromatophore indices for each three-hour period were summed and three subtracted from the total. The percentage of the total that each three-hour period occupied was then calculated.

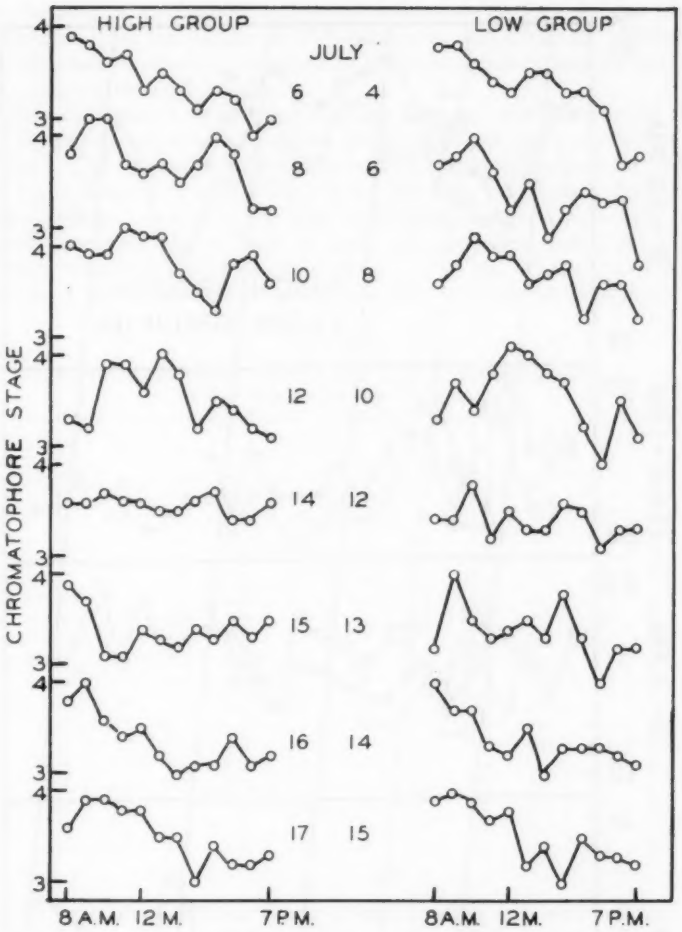

FIGURE 3. Daily pattern of melanophores of *Uca pugilator* from Series A (high group) and B (low group) on selected days.

Analysis of the data in the manner described above revealed a two-day difference, relative to the time of day, in the phases of the tidal rhythms of specimens of Series A and B and Series C and D through July 26 as indicated by the horizontal bars in Figures 4 and 5. For any given hour of the day the groups closer to the low tide mark exhibited corresponding maxima and minima two days earlier than the crabs from burrows closer to the high tide mark.

Analysis in the same manner of the data obtained with *Uca pugilator* during the summer of 1955 revealed regularly recurring 14.8-day cycles (Fingerman, 1956).

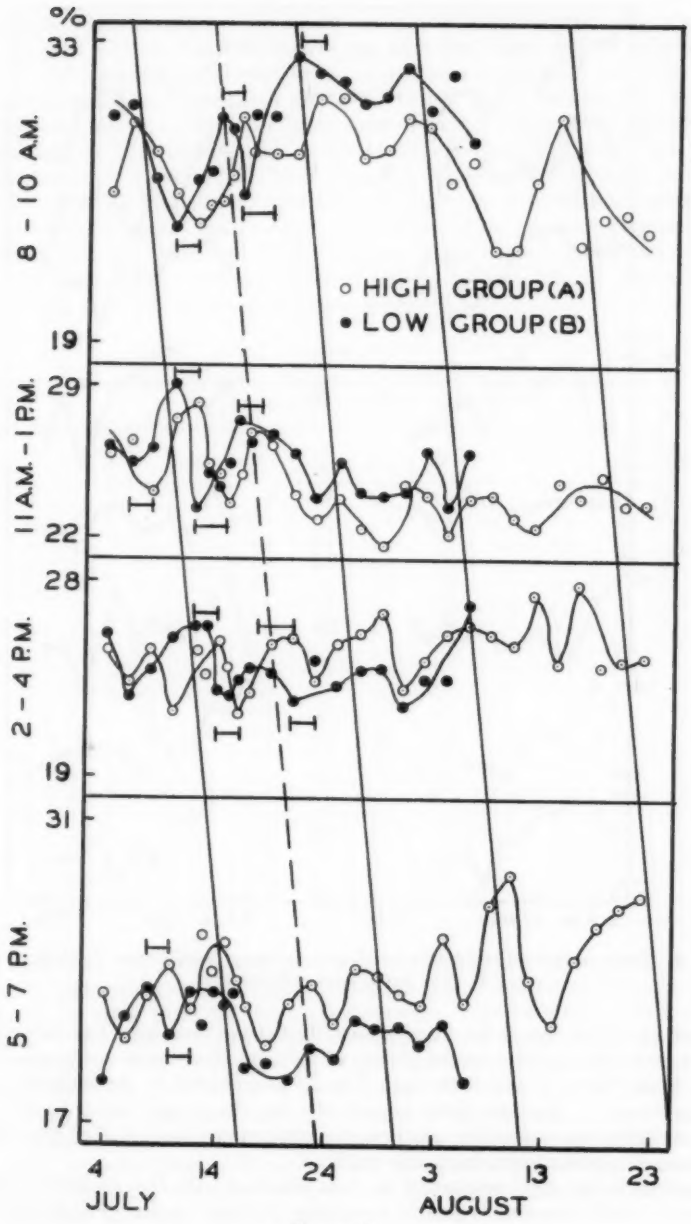
%
33
19
8 - 10 A.M.
HIGH GROUP (A)
LOW GROUP (B)
29
22
11 A.M. - 1 P.M.
28
19
2 - 4 P.M.
31
17
5 - 7 P.M.
4
14
24
3
13
23
JULY
AUGUST

FIGURE 4.

However, because of the absence of afternoon maxima through August 26 in the data collected in 1956, the pattern of 14.8-day cycles was different. Furthermore, the phase difference evident through July 26, 1956, disappeared and the four groups of crabs became rhythmically similar.

Inspection of Figure 4 reveals that the first 14.8-day cycle (shown by the first two solid diagonal lines) consisted of two 7.4-day rhythms as shown by the broken diagonal line. The diagonals were drawn midway between the two-day difference of Series A and B and C and D. The 7.4-day rhythms were due to the absence of late afternoon peaks. Instead of the tidal rhythm progressing across noon and into the afternoon the peak shifted back to early morning, thus producing a 7.4-day rhythm rather than the typical 14.8-day rhythm. Only two 7.4-day cycles were evident in Series A and B and were followed by a cycle of different frequency. A 7.4-day cycle was not evident in Series C and D because when they were collected Series A and B crabs were completing their second and last 7.4-day cycle. Furthermore, the two-day phase difference between the tidal rhythms of the crabs of Series C and D was not evident for longer than a week because of the unexpected loss of the phase difference that occurred in the four series of crabs about July 26.

TABLE III

The average melanophore index for each of the 12 daily observations of the Uca pugilator animals of Series C

		8 A.M.	9	10	11	12 M.	1 P.M.	2	3	4	5	6	7
July	19	3.7	4.0	3.5	3.8	4.0	4.0	3.5	3.9	3.4	3.4	3.4	2.6
	21	4.0	3.9	3.7	3.6	4.0	3.6	3.9	3.8	3.4	3.5	3.8	3.1
	23	3.4	4.3	4.2	3.6	4.0	3.8	4.1	3.7	3.7	3.3	3.8	3.6
	25	3.7	4.0	4.1	3.6	4.2	4.2	3.8	3.7	4.3	4.0	3.6	3.4
	27	3.7	4.1	3.7	4.1	3.7	3.4	4.0	3.8	3.6	4.1	3.8	3.7
	29	4.1	4.1	3.8	3.8	3.8	3.2	3.5	3.8	3.5	3.9	3.6	3.6
	31	4.1	4.0	4.0	4.0	3.2	3.1	3.3	3.0	3.2	2.9	3.2	2.5
August	2	4.0	3.4	4.1	3.4	4.0	3.2	3.1	2.8	3.0	3.1	3.3	3.2
	4	3.3	3.0	3.3	3.6	3.2	2.7	3.0	3.2	3.1	2.7	2.6	2.7
	6	2.8	3.1	3.4	3.6	3.5	3.4	3.4	3.5	3.7	3.3	3.1	2.6
	8	2.6	3.0	3.1	3.0	3.2	2.9	3.1	3.4	3.5	3.2	3.6	2.8
	10	2.9	2.9	2.7	2.6	3.1	2.5	2.5	2.6	2.5	3.3	3.0	3.5
	12	3.2	3.0	3.7	2.9	2.8	2.8	2.9	3.2	2.8	3.0	2.8	2.8
	14	2.9	2.7	3.4	2.7	2.6	2.7	2.4	2.4	2.1	2.5	2.8	2.7
	16	2.9	3.2	3.2	2.9	2.8	2.9	2.5	2.9	3.0	3.1	3.0	2.4
	18	3.0	3.0	3.4	3.1	2.8	2.7	2.7	3.1	3.0	2.9	3.1	3.1
	20	2.8	3.1	3.1	3.0	3.2	3.1	3.2	3.2	3.4	3.3	3.0	2.9
	22	3.0	2.9	3.0	3.0	3.2	3.3	3.3	3.4	3.6	3.4	3.3	3.5

When the phase difference disappeared a rhythm appeared with a frequency that had not been observed previously. Instead of a typical 14.8-day cycle, an 11.5-day rhythm appeared (Figs. 4 and 5). As evident from inspection of Tables I, III, and IV, maxima occurred in the afternoon on August 8. From the latter date until the

FIGURE 4. Relationship between percentage of daily melanin dispersion of *Uca pugilator* collected July 3 that occurred at each of four periods during the day and the day of the month. Circles represent fiddler crabs from the burrows closer to the high tide mark (Series A). Dots represent fiddler crabs from the burrows closer to the low tide mark (Series B).

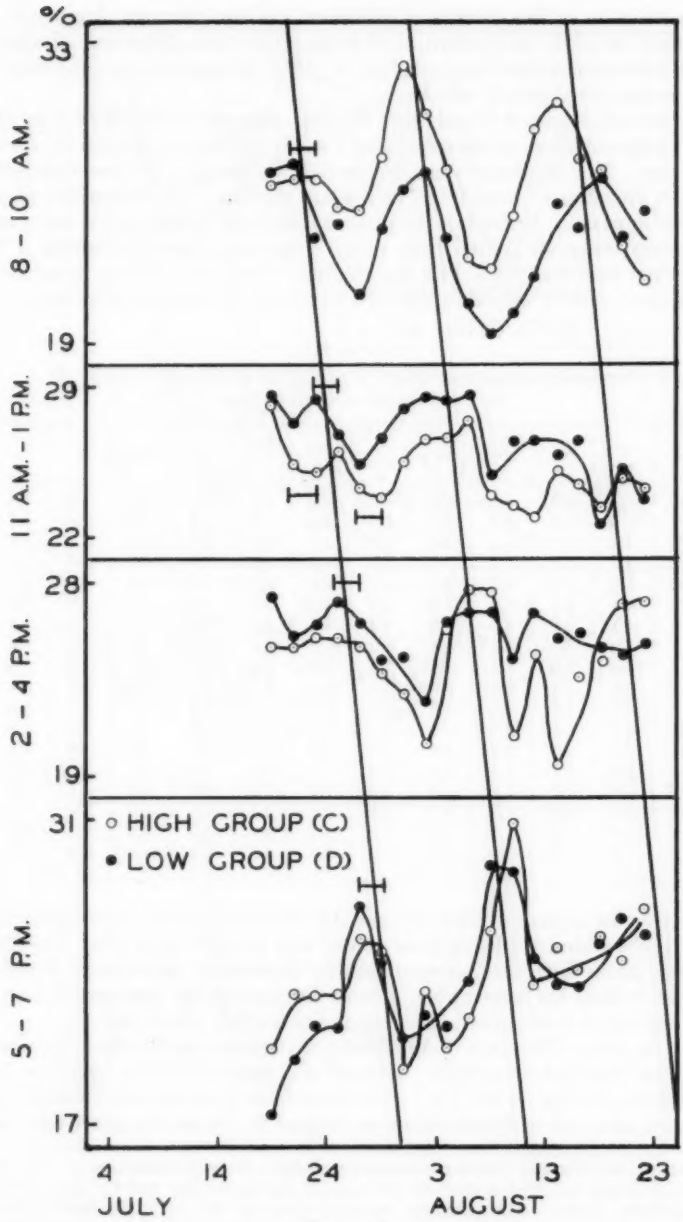
%
33
19
8 – 10 A.M.
29
22
11 A.M. – 1 P.M.
28
19
2 – 4 P.M.
31
17
5 – 7 P.M.
○ HIGH GROUP (C)
● LOW GROUP (D)
4
14
24
3
13
23
JULY
AUGUST

FIGURE 5.

observations of Series A, C, and D ended on August 22, the daily patterns exhibited a typical tidal progression of the peak across the day as observed with *Uca pugilator* during the summer of 1955. A typical 14.8-day cycle followed the 11.5-day cycle concomitant with the gradual progression of the tidal maximum across the day.

The relationship between the times of high and low tide (Fig. 2) and the maximum of the chromatophore readings determined each day of observation could not be determined with accuracy, since at the start of the observations the maxima of the chromatophore readings did not progress across the day but were confined to the morning hours. On some days the maximum would occur later than the time of

TABLE IV

The average melanophore index for each of the 12 daily observations of the Uca pugilator crabs of Series D

		8 A.M.	9	10	11	12 M.	1 P.M.	2	3	4	5	6	7
July	19	3.1	3.0	3.7	3.4	3.5	3.3	3.5	3.4	3.0	3.0	2.6	1.8
	21	3.2	3.7	3.6	3.8	3.2	3.5	3.4	3.3	3.3	3.3	2.8	2.4
	23	2.3	3.0	3.7	3.7	3.3	3.1	3.2	3.2	3.1	3.1	2.7	2.6
	25	2.7	3.5	3.8	3.8	3.3	3.5	3.6	3.4	3.7	3.1	3.1	2.9
	27	2.1	3.2	2.9	3.1	3.1	3.0	3.2	3.0	3.2	3.2	3.3	3.1
	29	2.2	3.4	3.5	3.3	3.3	3.1	3.0	3.1	3.0	3.1	3.2	2.9
	31	2.9	3.5	3.6	3.8	3.4	3.3	3.2	3.1	3.3	2.8	3.1	2.7
August	2	2.5	3.3	3.4	3.2	3.3	3.1	3.0	2.7	2.5	2.7	2.6	2.8
	4	2.4	2.8	3.1	3.2	3.1	3.0	3.1	2.8	2.9	2.8	2.8	2.2
	6	2.0	2.7	3.3	3.4	3.2	3.3	3.4	3.1	2.9	3.3	2.9	2.5
	8	1.6	3.0	2.8	2.8	3.0	2.8	3.0	3.1	2.9	3.1	3.2	3.2
	10	2.0	2.0	3.1	3.1	3.2	3.2	2.9	3.0	3.1	3.4	3.5	3.1
	12	2.4	3.0	3.5	3.3	3.4	3.4	3.3	3.4	3.4	3.3	3.2	3.1
	14	2.7	3.1	3.4	3.1	3.1	3.1	3.3	2.9	3.0	3.1	2.9	2.7
	16	2.9	2.9	2.6	3.1	2.9	2.9	2.8	2.9	3.0	2.7	2.9	2.6
	18	3.1	2.7	3.1	2.9	2.5	2.6	2.7	2.8	3.0	2.9	2.8	2.9
	20	2.8	2.8	3.0	2.9	2.8	3.2	2.9	2.9	3.0	3.1	3.1	3.0
	22	2.9	2.9	2.8	2.9	2.6	2.8	2.9	2.9	2.8	3.0	2.9	2.8

the tide and on other days earlier since the tides occurred at all hours of the 24-hour day but the maxima were restricted to the morning hours.

The relationship between the maximum of pigment dispersion and the time of tide from day to day must have been different at the end of the period of observation than at the beginning because of the 11.5-day cycle that intervened between the two 14.8-day cycles shown in Figure 4. The tides progressed at the typical semilunar rate of 14.8 days throughout the period of observation so that the phase relationship must have changed by 3.3 days, equivalent to 2.7 hours in a tidal cycle, in addition to the complication imposed by the appearance of typical cycles and maxima in the afternoon.

FIGURE 5. Relationship between percentage of daily melanin dispersion of specimens of *Uca pugilator* collected July 18 that occurred at each of four periods during the day and the day of the month. Circles represent fiddler crabs from the burrows closer to the high tide mark (Series C). Dots represent fiddler crabs from the burrows closer to the low tide mark (Series D).

The disappearance of the phase difference between the groups of crabs was in all probability due to some exogenous factor and not to removal of the crabs from direct contact with tides. If the latter explanation were correct, then crabs collected July 3 (Series A and B) would have lost their phase difference and taken on an 11.5-day cycle two weeks prior to the animals of Series C and D collected July 18. Since the crabs were not exposed in the laboratory to any overt stimuli capable of evoking the 7.4- and 11.5-day cycles observed during the summer of 1956, some exogenous factor might be considered as the mediating agent although natural rhythms have neither 7.4- nor 11.5-day cycles. Brown, Bennett and Ralph (1955) obtained evidence for a reversible effect of cosmic ray showers upon the responses of the chromatophore system of the fiddler crab *Uca pugnax*. The response of crabs exposed to increased intensity of cosmic ray showers, as compared with controls, was decreased pigment dispersion during the start of the day phase of the endogenous 24-hour cycle and increased dispersion during most of the remaining hours of the day.

Brown and Stephens (1951), in an investigation of the influence of length of photoperiod upon the amplitude of the daily pigmentary excursion of *Uca pugnax*, found that the longer the daily photoperiod the greater was the amplitude of the daily pigmentary excursion after the crabs were placed in constant darkness. Crabs with their burrows close to the low tide mark experience a shorter daily photoperiod than do animals whose burrows are close to the high tide mark. When the water covers the burrows the crabs are in darkness and emerge when the burrows are uncovered by the receding water. Burrows close to the low tide mark are uncovered later than burrows near the high tide mark and are covered earlier each day. Therefore, crabs living in burrows close to the low tide mark experience a shorter photoperiod each day than do crabs in burrows near the high tide mark.

The averages of the daily totals obtained for the first 33 days the crabs of Series A and B were in darkness were 40.96 and 37.59, respectively; averages for the first 34 days the crabs of Series C and D were in darkness were 39.65 and 36.62, respectively. The values for the crabs closer to the low tide mark (Series B and D) were less than the values for the crabs from the burrows close to the high tide mark (Series A and C).

An influence of change in day-length is also evident from inspection of the averages of the amplitude values. The time from sunrise to sunset in New Orleans was nine minutes less on July 18, 1956, than on July 3, 1956. The amplitude values for groups of crabs from the same set of burrows likewise decreased. Analysis of the daily amplitudes obtained with *Uca pugilator* during the summer of 1955 (Fingerman, 1956) also reveal an effect of change in photoperiod upon the amplitude of the daily pigmentary dispersion. On June 15, 1955, the time from sunrise to sunset was seven minutes more than on June 1, 1955. The average amplitude for *Uca pugilator* from the same set of burrows for the first 34 days in the laboratory was 37.75 for those collected on June 1, 1955, and 39.22 for those collected June 15, 1955. In this instance the day-length had increased between the first and second collection and the amplitude was consequently greater.

General Discussion

The results presented above support the conclusions of Fingerman (1956) concerning the tidal rhythms of color change of the fiddler crab *Uca pugilator*, as

well as provide new information about the nature of the rhythm. The time the burrows are uncovered at a particular level of the beach, and not the time of actual low tide on the beach, appears to be the primary determinant of the phases of the tidal rhythm relative to the 24-hour rhythm of color change.

Chromatophore rhythms with frequencies other than 12.4 and 24.0 hours and 14.8 days have not been observed previously. In the present investigation 7.4- and 11.5-day rhythms were described. The latter two, however, did not persist for more than one or two cycles. Evidently, the center or centers of rhythmicity of the fiddler crab can be set at one of several frequencies, some persistent and others not. Brown, Webb and Bennett (1955) have shown that *Uca pugnax* has the endogenous ability to mark off periods of solar and lunar day lengths in the absence of all possible rhythmic external signals. The persistent frequencies because of their correlation with frequencies of environmental events are probably of adaptive significance. On the other hand, no cosmic phenomenon has a 7.4- or 11.5-day frequency nor do fiddler crabs carry on activities correlated with these frequencies. The non-persistent rhythms of 7.4 and 11.5 days may have been imposed upon the crabs by exogenous factors and when these factors were no longer able to express themselves the crabs returned to the usual rhythm of 14.8-day frequency.

Summary and Conclusions

1. The tidal and semilunar rhythms of color change of the fiddler crab *Uca pugilator* have been subjected to further analysis.

2. The phases of these rhythms appear to be set according to the time the burrows of the crabs begin to be uncovered by the receding water following a high tide and not the time of actual low tide on the beach.

3. The amplitude of the daily pigmentary dispersion is also influenced by the time when the burrows are covered and uncovered. Crabs in burrows close to the high tide mark experience a longer daily photoperiod and consequently exhibit a greater daily amplitude of pigment dispersion.

4. A two-day phase difference, equivalent to 1.6 hours in a tidal cycle, was found between two groups of crabs collected from two discrete sets of burrows different distances from the high tide mark. The tidal maximum of pigment dispersion at any given hour of the day occurred two days earlier in the crabs collected closer to the low tide mark.

5. Measurements of the beach where the crabs were collected revealed that the receding water begins to uncover the burrows close to the high tide mark 1.6 hours earlier than the burrows closer to the low tide mark begin to be uncovered, the same difference observed in the laboratory.

6. The daily patterns of pigment dispersion were different during the first two weeks of observation from the patterns observed in previous investigations. Maxima of pigment dispersion did not occur in the late afternoon during the first three weeks of observation but were restricted to the morning hours. The absence of peaks in the afternoon resulted in cycles with a 7.4-day frequency.

7. An 11.5-day cycle also appeared and was followed by a typical 14.8-day cycle accompanied by maxima of the daily curves in the late afternoon.

8. The results are discussed in terms of possible exogenous causes of the 7.4- and 11.5-day cycles.

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EVIDENCE FROM SEA URCHIN–SAND DOLLAR HYBRID EMBRYOS FOR A NUCLEAR CONTROL OF ALKALINE PHOSPHATASE ACTIVITY

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Several investigators (Hultin, 1948a, 1948b; Bohus Jensen, 1953; Tyler and Metz, 1955) have shown that hybridization in echinoids can be facilitated by treatment of the eggs with trypsin. A recent review by Moore (1949) has covered the problem of inheritance in hybrid plutei of echinoids. It seemed of interest to determine the activity of two enzymes (acid and alkaline phosphatase) in the developing embryos of two separate species and of the hybrids. In the hybrids it was hoped that it would be possible to assess the relative role of the nuclei and the cytoplasm in directing the synthesis and activity of these two enzymes.

Alkaline phosphatase activity rises rapidly from gastrulation on to the pluteus stage (Mazia *et al.*, 1948; Gustafson and Hasselberg, 1950), while acid phosphatase maintains a constant level of activity during sea urchin development according to Gustafson and Hasselberg (1951). However, the latter authors have noted a slight but definite rise in acid phosphatase activity in the sea urchin *Paracentrotus lividus*.

Paternal antigens arising by the late blastula stage in sea urchin hybrids have been demonstrated serologically by Harding, Harding and Perlmann (1954), but little information exists dealing with quantitative chemical differences in hybrids. Brachet (1954) has expressed the belief that the nucleus exerts a very important control over enzymes of the microsomes, as evidenced from work upon nucleated and enucleated halves of amoebae.

Utilizing the Gomori–Takamatsu cytochemical technique Krugelis (1947b) has noted that nuclear alkaline phosphatase activity increases from gastrulation to the pluteus stage while that of the cytoplasm declines. In the unfertilized egg the weak staining reaction for the enzyme is primarily located in the cytoplasm (Krugelis, 1947a) and this is borne out by Mazia *et al.* (1948) who utilized biochemical methods and found equal activity in nucleated and enucleated halves of unfertilized eggs separated by the Harvey method. The Gomori-Takamatsu technique has been criticized by Novikoff (1951) and Johansen and Linderstrom-Lang (1952); these authors believe that diffusion obscures the accurate localization of the calcium phosphate precipitate and that nuclei may adsorb the precipitate. Novikoff *et al.* (1950) isolated rat liver nuclei and found less alkaline phosphatase activity in that fraction as compared to the cytoplasm, and Stern *et al.* (1952), utilizing a non-aqueous medium for isolating nuclei (Behren's technique), obtained similar results for nuclei isolated from the thymus gland. However, Dounce (1943) found a higher con-

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centration of alkaline phosphatase in the isolated nuclei from rat liver as compared to the whole tissue. Danielli (1953) expresses the belief that the Gomori-Takamatsu cytochemical technique can successfully localize alkaline phosphatase in the cell and this would mean that a number of workers who cite a nuclear localization of alkaline phosphatase (Danielli, 1946; Krugelis, 1947b; Brachet and Jeener, 1948; and Bradfield, 1950) are correct. The question of localization of this enzyme is an open one and it was hoped the method of hybridization might help to clarify it.

It was originally planned to hybridize reciprocally *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus* (see Moore, 1943, for a discussion of maternal and paternal inheritance in this cross), but it was found that there was essentially no difference in acid and alkaline phosphatase activity between the two species. Also it is known that when *S. franciscanus* eggs are fertilized by *S. purpuratus* sperm, development is blocked at the late blastula stage (Moore, 1943). Hybridization of *S. purpuratus* and *Dendraster excentricus* is advantageous in that these genera are more distantly related; they differ markedly in their speed of development, and the cross can be made reciprocally.

Materials and Methods

Eggs and sperm of *Dendraster* and *S. purpuratus* were obtained by injection of 0.5 *M* KCl (Tyler, 1949) into the body cavity. The eggs of *Dendraster* could be fertilized by the usual dilute suspension of *S. purpuratus* sperm used in the homologous crosses. In order successfully to fertilize *S. purpuratus* eggs with *Dendraster* sperm (Tyler and Metz, 1955), the eggs were placed in a 0.05% trypsin (crystalline-lyophilized preparation from Worthington Biochemical Sales Co.) solution for ten minutes and the eggs were then washed several times with sea water to remove the trypsin. They were then fertilized with an amount of 1% sperm (1 drop of dry sperm/5 cc. sea water) which was in forty-fold excess of the volume of sea water containing the eggs. After the eggs were left in this concentrated sperm solution for 30–40 minutes (with frequent agitation of the solution), the eggs were then washed four or five times to remove the excess sperm and cultured in a slowly rotating four-liter flask which floated in a tank of running sea water. The temperature of this running sea water was usually about 10° C. With this procedure about 40% of the eggs were successfully fertilized and hatched swimming blastulae could be separated from the unfertilized eggs.

On the first, second, third, and fourth days after fertilization embryos were collected by centrifugation and were washed several times with the appropriate buffer. These washes were carried out rapidly so as to prevent any loss of the enzyme. For the acid phosphatase assays this was a 0.1 *M* sodium acetate-acetic acid buffer of pH 5.32; for the alkaline phosphatase assays a 0.1 *M* sodium veronal-HCl buffer (0.0015 *M* $MgCl_2$) of pH 9.0 was utilized. For a given assay the embryos were suspended in 4 cc. of the acid or alkaline buffer and disintegrated in an all-glass homogenizer which was kept immersed in an ice bath during homogenization. Then a two-cc. aliquot of the brei was added to two cc. of the substrate (0.1 *M* sodium β glycerophosphate); for the acid phosphatase assays the pH of the substrate solution was adjusted to 6.0. One of the mixtures of brei plus substrate was immediately inactivated by the addition of 0.8 cc. of 60% trichloracetic acid while the other was allowed to incubate at 25° C. for a two-hour period at which time a simi-

lar amount of trichloracetic acid was added. The control and experimental samples were then centrifuged at approximately 10,000 G and the trichloracetic acid supernates were collected. Phosphorous assays were made upon these samples utilizing the Fiske-Subbarow technique (1925) and the control values subtracted from the experimental ones. The trichloracetic acid precipitates were each suspended in one cc. of water and total nitrogen determinations (Umbreit *et al.*, 1948) were made upon aliquots from these samples. Acid or alkaline phosphatase activity is stated as the ratio of total micrograms of phosphate released in a two-hour period divided by the total micrograms of trichloracetic acid-insoluble nitrogen. Acid-insoluble nitrogen is a good standard for these measurements since it remains fairly constant during early development.

Results

Preliminary assays of acid phosphatase in developing embryos of *Strongylocentrotus purpuratus* and *S. franciscanus* showed the activity of this enzyme to be essentially similar in the two species, and preliminary determinations of acid phosphatase in *Dendraster* gave values within this same range. Since acid phosphatase could not be used quantitatively to distinguish *S. purpuratus* from *Dendraster*, this enzyme was not assayed in the hybrids nor were further determinations made in the homologous species. The series of determinations for any given batch of eggs seemed to indicate a slight increase in activity of acid phosphatase during development, but the average values only substantiated an increased activity from the blastula to the gastrula stage.

The activity of alkaline phosphatase was quite different in *Dendraster* and *S. purpuratus*, rising quite sharply for the sand dollar embryos and much more slowly for the sea urchin embryos (Fig. 1). The points in Figure 1 represent two series of experiments, all of which were conducted under similar conditions and gave excellent reproducibility. It was of interest to see if the activity of this enzyme in the hybrids was characteristic of the maternal or the paternal species, or if it might be intermediate between the two. If the latter alternative held true, this would be a quantative indication of a nuclear control of alkaline phosphatase activity, whereas a maternal rate of activity would indicate a cytoplasmic independence of this enzyme. It was not expected that a paternal activity rate would be found.

Hybridization of *Dendraster* eggs by *S. purpuratus* sperm results in normal development up through gastrulation but in the experiments reported here the embryos then became abnormal and gave rise to so-called spherical plutei. These "plutei" did not swim actively and died within a few days. It is believed that the low alkaline phosphatase activities which were found here are a reflection of the poor condition of the embryos. Even if these embryos had developed normally, this would not have been the best cross to ascertain nuclear or cytoplasmic dependence of the enzyme since the maternal species (*Dendraster*) normally has a high alkaline phosphatase activity by the pluteus stage. An intermediate value in this case would be a decrease from the normal activity of the maternal species and might be ascribed to lowered vitality of the hybrids. However, in the *S. purpuratus* ♀ × *Dendraster* ♂ hybrids an intermediate value would be an increase from the normal activity of the maternal species and could clearly be ascribed to the effect of the paternal nuclear material during development. It was fortunate that these hybrids

developed perfectly normally. Some were kept as long as 12 days after fertilization and they were still perfectly healthy plutei at that time.

The *S. purpuratus* ♀ × *Dendraster* ♂ hybrids were strictly maternal in terms of rate of development both before gastrulation and up to the pluteus stage; *Den-*

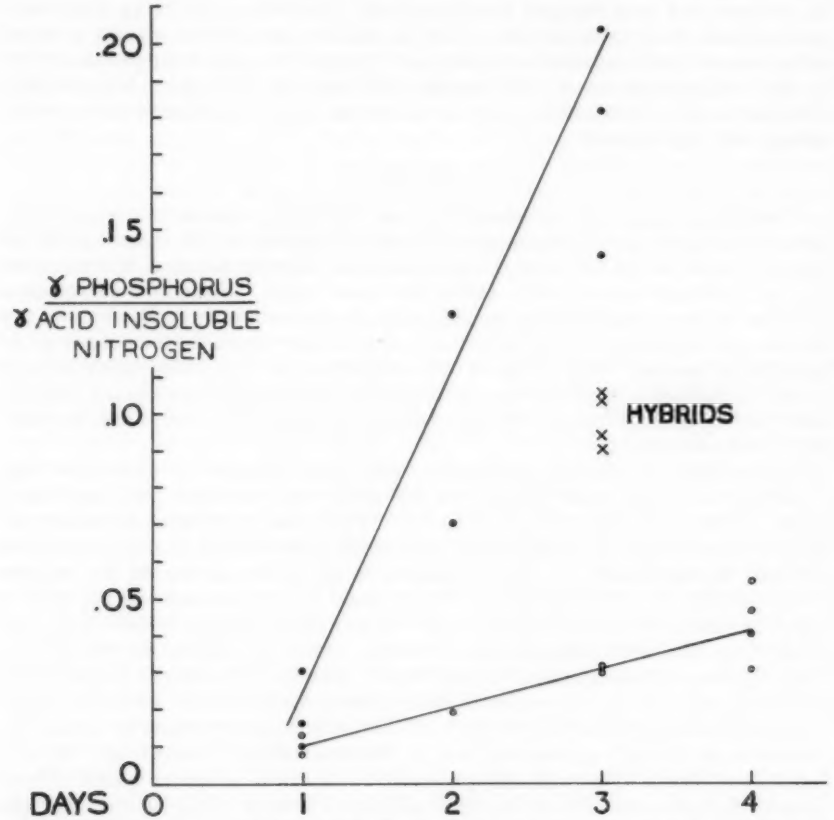

FIGURE 1. Alkaline phosphatase activity of *Strongylocentrotus purpuratus*, *Dendraster excentricus* and *Strongylocentrotus purpuratus* ♀ × *Dendraster excentricus* ♂ hybrids. Expressed as the ratio of micrograms phosphorus released in a two-hour period per microgram of acid-insoluble nitrogen. Solid dots indicate determinations upon *Dendraster*, open circles are for *S. purpuratus* and × indicates determinations upon the hybrids.

draster reaches the pluteus stage three days after fertilization whereas *S. purpuratus* takes four days. The day after fertilization (first day) the hybrids were early blastulae, the next day (second) they were hatched swimming blastulae or early gastrulae, the third day they had reached the prism stage and on the fourth day after fertilization they were plutei. In terms of morphological appearance the hybrid

plutei look very much like those of *S. purpuratus*. They lack the extended oral and anal arms of the *Dendraster* plutei and the skeletal rods are located as they are in *S. purpuratus* plutei.

Alkaline phosphatase determinations in the *S. purpuratus* ♀ × *Dendraster* ♂ hybrids were routinely made on the third day after fertilization when the hybrids had reached the prism stage. Assays were made at this time (third day) since no estimations were made upon *Dendraster* plutei after this period, and also by this third day there was a marked disparity between the alkaline phosphatase activity of the two homologous species which is not true at the earlier stages when alkaline phosphatase activity of the two species is more similar. The enzyme estimations revealed values intermediate to those of either homologous species (Fig. 1) at a similar time after fertilization. These hybrid prism activities were not only greater than those of *S. purpuratus* prisms but also distinctly higher than the values obtained for four-day *S. purpuratus* plutei. This indicates that alkaline phosphatase activity is not merely reaching an appropriate level for a given stage of development, as is the case with so many metabolic activities. These data are interpreted as an elevation of alkaline phosphatase activity due to the presence of *Dendraster* nuclear material in the developing embryos. It is not known if this nuclear stimulation of alkaline phosphatase indicates a nuclear localization of the enzyme, but it certainly seems to demonstrate a quantitative nuclear control for this enzyme with the intermediate value resulting from the action of both maternal and paternal nuclear elements.

Several attempts to induce higher acid and alkaline phosphatase activities in *S. purpuratus* embryos were carried out by adding sodium β glycerophosphate (final concentration 0.1 *M*) to several of the cultures at the mesenchyme blastula stage. The blastulae were allowed to develop for two days in the sea water containing the substrate and then acid and alkaline phosphatase assays were made at the pluteus stage. However, in two sets of experiments involving such cultures and their controls (where no substrate was added) no increase in activity was noted. Usually the activity of the controls was slightly higher than that of the cultures to which glycerophosphate had been added.

In order to determine if inadequate homogenization might account for the low alkaline phosphatase values, *S. purpuratus* embryos were frozen and thawed several times before homogenization so as to facilitate rupture of the cells. This procedure had no effect upon alkaline phosphatase activity since similar values were obtained for fresh material, once frozen, and twice frozen samples. Microscopic examinations of the homogenates were made routinely in all experiments. In all cases no large clumps of cells were observed, but undoubtedly not all cells were broken by the homogenization. This does not affect the interpretation of the results since the embryos of *S. purpuratus*, *Dendraster* and the hybrids were disintegrated in the same manner, and an equal degree of homogenization apparently was obtained in each case. It might also be expected that the hybrids would yield homogenates much like those of *S. purpuratus* since the only *Dendraster* component of the hybrids is nuclear and hence they are probably of equal fragility.

The author wishes to express his gratitude to the staff of the Friday Harbor Laboratories for their many kindnesses during the summer.

SUMMARY

1. Alkaline phosphatase estimations upon sea urchin embryos (*S. purpuratus*) showed a slow rate of increasing activity up to the pluteus stage while sand dollar embryos (*Dendraster excentricus*) showed a very rapid increase up to this same stage.

2. Hybridization between *Strongylocentrotus purpuratus* ♀ and *Dendraster excentricus* ♂ resulted in alkaline phosphatase activity at the prism stage which was intermediate between that of the two homologous species and higher than that of the maternal species. This is interpreted as indicating a nuclear control of alkaline phosphatase activity.

3. Addition of substrate (0.1 *M* sodium β glycerophosphate) to cultures of developing *S. purpuratus* embryos did not affect an increase in acid or alkaline phosphatase activity.

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OBSERVATIONS ON OSMOREGULATION IN THE ARCTIC CHAR (SALVELINUS ALPINUS L.)[1]

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The different groups of euryhaline fishes have developed somewhat different mechanisms for maintaining the relative constancy of the concentration of their "milieu interieur" in the face of large changes in the external osmotic pressure. Following such an external change, many change their internal concentrations only transitorily, in the same direction as the external variation. A return to essentially the original conditions usually follows shortly (in adult female eels (*Anguilla*): Boucher-Firly, 1935; Duval, 1925; in sticklebacks (*Gasterosteus*); Gueylard, 1924; Koch and Heuts, 1943; in killifish (*Fundulus*): Burden, 1956). Anadromous salmonid fishes, however, have long been known to regulate their blood concentrations on two distinct levels (probably the end-points of an acclimation curve). In almost all such salmonids studied so far, the transition from salt to fresh water (or the reverse) is accompanied by a fall (or rise) in total blood concentration of about 25% (the Atlantic salmon, *Salmo salar*, and the Chinook salmon, *Oncorhynchus tshawytscha*, however, change by only 12% (Fontaine and Koch, 1950; Greene, 1926)). Plasma freezing point depressions vary from species to species, being 0.67–0.90° C. in salt water, 0.55–0.72° C. in fresh water (Benditt *et al.*, 1941; Fontaine, 1943, 1948; Fontaine, Callamand and Vibert, 1950; Fontaine and Koch, 1950; Greene, 1904; Kubo, 1953).

The Arctic char (*Salvelinus alpinus* L.) is an anadromous salmonid fish common in fresh and coastal salt waters throughout most of the Arctic. As in other sea-going char, its migrations from fresh to salt water and back again are somewhat different from those of other salmonids in that its winter periods in fresh water lakes and rivers are long compared to its summer periods in the ocean, rather than vice versa (Andrews and Lear, in press; Backus, 1952; Sprules, 1953). Osmoregulation in this form has not been studied, probably due to its inaccessibility. The present paper describes some observations made on the osmoregulatory abilities of adult Arctic char on their return (spawning) migration to fresh water after a summer in the ocean.

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[2] The author's thanks for aid and cooperation are due the following people: In Labrador (1954 "Blue Dolphin" Expedition), Cmdr. D. C. Nutt, Drs. P. F. Scholander and L. van Dam, and P. Hay. At Churchill, Manitoba, Mr. P. Bussadore, Mr. and Mrs. A. MacIver, Mr. and Mrs. I. Smith, and Dr. D. E. Sergeant. Mr. C. L. Claff kindly permitted the use of his flame photometer. Dr. van Dam performed the plasma chloride determinations at Hebron.

The material is of a preliminary nature in many ways, but serves to show that the Arctic char is similar to other salmonid fishes in regulating its blood concentration on two levels. Agreement between experimental results and data obtained from fish living in fresh and salt waters provides a basis for further experimental study of osmotic phenomena during the migrations of these fish. Some data on regulation of muscle concentrations are also presented. Differences in results obtained from char in northern Labrador and Hudson Bay indicate the possibility of physiologically different populations in this species.

Materials and Methods

Regulation of total plasma concentration, chloride and potassium, and of total muscle solids, chloride, and potassium was studied in mature adult char of both sexes by means of observations on fish living in salt or fresh water, and by time series of observations immediately following direct transfers of fish from salt to fresh water. Conditions of temperature, stage in life cycle, etc., were kept fairly constant in these experiments. Char were taken by means of gill nets from Hebron Fjord, Labrador, and Hudson Bay near Churchill, Manitoba, during late July and early August of 1954 and 1955, respectively.

Seven char were used to establish the normal salt water ranges for plasma freezing point and chloride concentration in fish in Hebron Fjord (water of 28.6 ‰ salinity, 9° C. temperature). Five Churchill char were used similarly, observations on these consisting of plasma chloride and potassium concentrations, and total muscle solids, chloride, and potassium concentrations (Hudson Bay water was of approximately 26 ‰ salinity, 10–12° C. temperature).

Fresh water ranges for plasma freezing point and chloride concentration were determined at Hebron in several small land-locked char from a small lake, in some pre-sea-run parr from a river, and in an adult char that had returned to fresh water by itself. No fresh water char were obtainable at Churchill.

The osmotic stresses these fish undergo during the course of their migrations from the sea were approximated by transferring four Hebron fish and seven Churchill fish directly from salt to fresh water. Temperatures at Hebron were: salt water, 9°, fresh water, 5–10°; at Churchill: salt water, 10–12°, fresh water 14–16°. Changes in plasma freezing point with time were followed in three of the Hebron fish via serial blood samples taken at intervals up to 77 hours following transfer. The fourth fish was sampled initially and after 77½ hours. Two of the Hebron char were then returned to the Fjord and sampled again following death (after some fifteen hours in salt water).

Only two of the Churchill char survived for more than one hour after transfer from salt to fresh water—these for two and six hours, respectively. Blood and muscle concentrations were determined in these, after the periods mentioned, as in the salt water fish. The approximately 5° C. thermal shock to which these fish were subjected, combined with a lack of running fresh water, hence a need for aeration by hand dipping, probably explains in great part the lowered survival as compared with the Hebron fish. The speed with which death followed transfer makes it seem unlikely that this is the complete explanation, however.

Blood samples were taken via heart puncture from all fish. The samples were heparinized, centrifuged, and the plasma pipetted off. Plasma freezing points

were determined at Hebron using the method of Pounder and Masson (1934) modified for field use (Scholander *et al.*, in press). Plasma chloride determinations were made using the method of Schnohr (1934). Plasma potassium concentration was determined on the Churchill samples following dilution with Pyrex-distilled water on a Baird Associates internal standard flame photometer. Probably due to coagulation of the proteins in these samples, which may have interfered with activation of the ions by the flame, only three of these analyses gave what appear to be reasonable values.

Four samples of tissue from the dorsal muscle mass, averaging about 100 mg. wet weight, were also taken from each of the Churchill fish. Total solids were determined by weighing these before and after complete drying in an oven at 105° C.

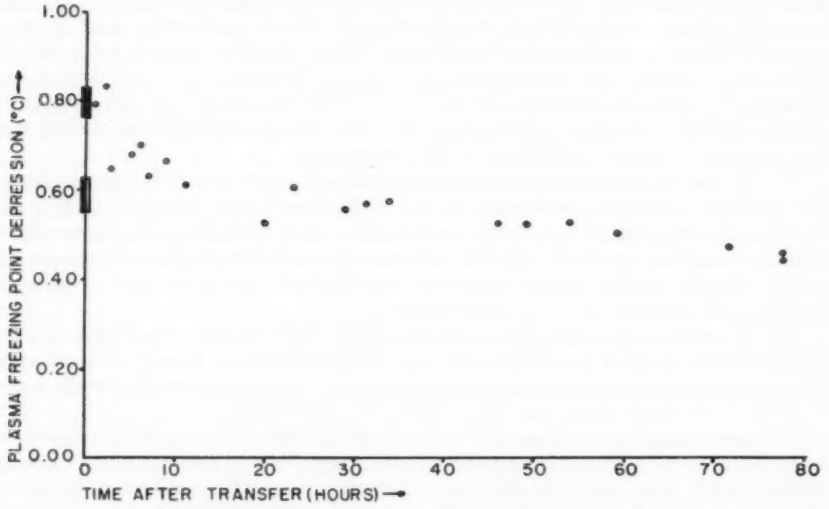

FIGURE 1. Changes of plasma freezing point depression with time following direct transfer from salt to fresh water of Arctic char (*Salvelinus alpinus* L.) from Hebron Fjord, Labrador. Ranges for fish naturally acclimated to salt water (black bar) and fresh water (black and white bar) indicated on ordinate. Temperature 5–10° C.

Following digestion with concentrated nitric acid and 30% hydrogen peroxide, duplicate chloride and potassium analyses were carried out. Methods were as above.

Precision of the freezing point determinations is ± 0.05° C. Plasma chloride analyses agreed within 1%, as did total muscle solids. Muscle chloride and potassium duplicates agreed within 5%.

RESULTS AND DISCUSSION

Figure 1 shows the behavior of total blood concentration in control and experimental char from Hebron Fjord. The total plasma concentration in Hebron char living in fresh water is about 25% lower than the salt water concentration. The char is thus like most other anadromous salmonids in this regard. Beginning

two hours after transfer from salt to fresh water, the experimental fish also decreased their plasma concentrations by about 25% over a period of approximately twenty hours. They then became fairly stable. It therefore seems that sudden transfer experiments duplicate at least some of the physiological events occurring during the migrations of these fish. This last might have been expected, since individual salmonids frequently make the transition from salt to fresh water and back again as rapidly as they can swim through the estuaries involved (Benditt *et al* 1941; Greene, 1910; Killick, 1955).

The continuing decrease in plasma concentration below the normal fresh water level which occurred in the experimental fish after about 35 hours in fresh water could have been the result of several influences. First, the fish were not fed; second, they may well have lost salt via their urine as a result of having been handled

TABLE I

Blood concentrations in arctic char

Source of fish	Plasma Δ_F (° C.)	Plasma $[Cl^-]$ (meq./l.)	Equivalent Cl^- Δ_F (° C.)*	Plasma $[K^+]$ (meq./l.)
Hebron Fjord, salt water	0.80	148	0.28	
	0.80	191	0.36	
	0.81	191	0.36	
	0.82	213	0.40	
	0.82	214	0.40	
	0.83			
	0.76			
Hebron Fjord, fresh water (land-locked)	0.59	144	0.27	
	0.61	144	0.27	
	0.63	161	0.30	
Churchill, salt water		177	0.33	5
		153	0.28	8
		156	0.29	1
		130	0.24	1
		132	0.25	2
Churchill				
2-hr. transfer		134	0.25	1
6-hr. transfer		124	0.23	8

* Equivalent $Cl^-\Delta_F$ calculated from: $\Delta_F = 1.86\ [Cl^-]$

(laboratory diuresis of Grafflin, 1931, 1935, and Forster, 1953); and third, their skin permeability, hence rate of water uptake in a hypotonic medium, may well have been increased as a result of loss of slime during handling.

The two Hebron char transferred back to the Fjord after the experiment, after fifteen hours in salt water, had plasma freezing points of $-0.75°$ C. This is essentially the original salt water value.

Table I summarizes the data on blood concentrations obtained from both Hebron and Churchill control fish and Churchill transfers. With the exception of the first salt water char from Hebron, the calculated equivalent Cl freezing point is essentially a constant fraction of the total freezing point (45–50%). More exact regulation of the concentrations of other plasma components is thus indicated. Similar behavior of chloride and total concentrations has been noted in brook and brown trout (unpublished data of the writer and van Dam). Fontaine, Callamand and

Vibert (1950), however, found a decrease of only 4% in plasma chloride in Atlantic salmon (*Salmo salar*) when total concentration dropped 13%.

The Churchill material generally supports the Hebron results. Plasma chloride concentration in the Churchill fish in fresh water for six hours is approximately 18% lower than the mean plasma chloride concentration for Churchill char in salt water. Plasma freezing point behaves similarly in the Hebron char. Note, however, that plasma chloride concentrations in the Churchill fish are generally much lower than in the Hebron fish. The mean difference of 20% seems too large to be the result of acclimation to differing salinities (the salinities differing only by 10%.). The marked differences between Hebron and Churchill char with respect to survival following transfer were noted earlier. Even allowing for the poor conditions encountered at Churchill it seems likely that real physiological differences exist between these populations. Marked differences in growth characteristics differentiating these two groups (Andrews and Lear, in press; Backus, 1952; Sprules, 1953; unpublished data of the author) also make this seem likely (though differences in food supply might well account for this last).

TABLE II

Muscle concentrations in Churchill arctic char

Source of fish	Total muscle solids (gm./kg. wet weight)	Muscle $[Cl^-]$ (meq./kg. wet weight)	Muscle $[K^+]$ (meq./kg. wet weight)
Salt water	276	22	123
	224	20	130
	266	8	120
	226	8	120
	260	6	125
2-hr. transfer	242	4	127
6-hr. transfer	244	3	150

Table II indicates that the one Churchill fish surviving transfer for six hours regulated total muscle concentration very well. Muscle potassium, however, seemingly increased markedly. The concentrations of the same muscle components in brook and brown trout under similar conditions behave very differently, however (data of the author and van Dam). In these other forms there is a close parallelism between changes in muscle concentrations and changes in the blood. Further work on the char is obviously needed.

In closing it should be noted that the blood and muscle potassium concentrations reported by Jones (1956) for fresh water brown trout agree very well with the figures given in Tables I and II for the Churchill salt water char (low plasma potassiums excepted).

SUMMARY

1. Adult Arctic char (*Salvelinus alpinus*), taken in summer from Hebron Fjord, Labrador, and Hudson Bay near Churchill, Manitoba, were transferred directly from salt to fresh water under fairly constant conditions.

2. Decreases in blood freezing point and chloride concentration of the order of 25% were found, the char thus being like most other anadromous salmonids in this

respect. The possibility of much better regulation of muscle concentrations is indicated.

3. Data are presented on plasma freezing point, chloride, and potassium, muscle solids, chloride, and potassium.

4. Physiological differences between populations of char are indicated.

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A COMPARATIVE STUDY OF THE GILL AREA OF CRABS

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In a comparative study of the gill area of marine fishes (Gray, 1954), it was shown that a definite correlation exists between the size of the gill area, the degree of activity, and the habits of the fishes concerned. It was found that sluggish bottom-dwelling species have proportionately much less gill surface than do fast swimming pelagic fishes. In this paper an attempt is made to find out if similar correlations exist in crabs from different habitats. Several correlations pertinent to the present discussion have already been pointed out by others. Ayers (1938) indicated that intertidal and land crabs consumed oxygen at a higher rate than did the strictly aquatic species. Pearse (1929a, 1929b, 1950) in his study of the emigration of animals from the sea has reported that there is a lessening of gill volume as crabs emigrate toward land. Pearse determined only the gill volume, not the gill area. More recently Vernberg (1956) has shown in a series of crabs that oxygen consumption of the whole animal and of gill tissue is highest in terrestrial species and decreases progressively as the habitat approaches ocean depths.

This paper presents the results of a study of the gill areas of sixteen species of brachyuran crabs from six taxonomic families, and representing both pelagic and benthic species, and those living below the low tide level, those of the intertidal zone, and those that live out of water most of the time.

Grateful acknowledgment is made to the Duke University Research Council for partial support of this research and to Miss Darlene Connor and Miss Barbara Galloway for the technical assistance.

Materials and Methods

The method used in the determination of gill area in crabs was similar to that employed in the determination of gill area in fishes (Gray, 1954). With crabs, however, since the gill platelets are so much larger, the procedure is somewhat less tedious. Each crab was weighed after first removing surplus water from the body and gill chambers with paper toweling. The gills were then removed from one side and each placed in a separate Petri dish. The total number of platelets for each gill was counted under a dissecting microscope or computed after measuring the length of each gill with vernier calipers and determining the average number of platelets per millimeter of length. After preliminary trials to observe the range of sizes, what appeared to be average size platelets from each gill were removed and mounted in sea water on slides. Using a dissecting microscope, camera lucida drawings were made of the selected platelets and the area of these determined by means of a planimeter. Knowing the magnification used and the total number of platelets, the total gill area could be readily calculated. A weak point in the pro-

cedure is that it calls for judgment on the part of the observer in selecting average size platelets for the camera lucida drawings. However, the method seems no less accurate and is far less cumbersome than the mathematical methods employed by Riess (1881), Putter (1909), and Price (1931) in estimating the gill areas of fishes.

Gill areas were obtained for the following species, listed more or less in order from the most land-adapted to the strictly aquatic: the ghost crab, *Ocypode albicans;* the wharf crab, *Sesarma cinerea; Sesarma reticulata;* the fiddler crabs, *Uca minax, Uca pugnax,* and *Uca pugilator; Panopeus herbstii;* the stone crab, *Menippe mercenaria;* the spider crabs, *Libinia dubia* and *L. emarginata; Hepatus epheliticus;* and five portunids, the blue crab *Callinectes sapidus, Areneus cribarus, Ovalipes ocellatus ocellatus, Portunus gibesii,* and *Portunus spinimanus.*

The spider crab, *Libinia emarginata,* was obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts and collected from the north side of Cape Cod. All other species were collected in the vicinity of the Duke University Marine Laboratory, Beaufort, North Carolina.

Habits and Habitats of the Crabs

Of the crabs studied, the ghost crab, *Ocypode albicans,* is by far the most land-adapted. It may make its burrows in the dunes at considerable distances from the high tide mark. It spends very little time in the ocean and, in fact, cannot survive prolonged submergence. It can, however, withstand desiccation to only a very limited degree and in hot weather comes out mainly at night and feeds near the water's edge. The wharf crab, *Sesarma cinerea,* also spends most of its time on land, hiding out under the drift in the daytime and coming out to feed at the water's edge at night. Both *Ocypode* and *S. cinerea* are very active and move at a rapid rate when disturbed.

The fiddler crabs usually make their burrows near the high tide mark where they will be submerged for at least a portion of each day. The sand fiddler, *Uca pugilator,* is found in large numbers along the sandy protected beaches of the estuaries near the ocean. It appears from its burrows with the receding tide, migrates to the water's edge, and may be exposed on the moist sand of the beach for several hours. Of the fiddlers, *Uca minax* is farthest removed from the ocean, making its burrows in mud banks of the marshes and ditches, often quite far from the sea and where tidal effect is less than on the open beaches and where the salinity may be greatly reduced. It does not travel as far from its burrows as *U. pugilator* and is exposed for shorter periods of time. *Uca pugnax* occupies a somewhat intermediate habitat between that of *U. minax* and *U. pugilator* and tends to occupy the salt marshes. *Sesarma reticulata,* more robust and less active than *S. cinerea,* lives in mud banks with *Uca minax.*

Panopeus herbstii may be easily captured at low water in the lower intertidal zone of exposed oyster reefs, in crevices and under stones of rock jetties. The stone crab, *Menippe mercenaria,* can be found in crevices of rock jetties at and below the low tide level. *Panopeus* and *Menippe* are not as active as the fiddler crabs, but are much more active than *Libinia.*

Libinia dubia and *L. emarginata* are slow moving bottom species living well below the low tide level. These are the most sluggish of the crabs studied. In

marked contrast, another bottom-dwelling species, *Hepatus epheliticus*, is very active indeed and can dig rapidly in the bottom sand where it is prone to hide.

The portunid crabs are all active swimmers, quick in movement, and capable of rapid burrowing in the sandy bottom. The blue crab, *Callinectes sapidus*, invades both the open sea and the estuaries. *Areneus cribarus* frequents the area of surf along the outer beaches. *Ovalipes ocellatus*, *Portunus spinimanus*, and *P. gibesii* are small crabs commonly taken in shrimp trawls in 20 to 60 feet of water, but are also occasionally found in the estuaries.

Results and Discussion

Table I presents the gill areas and number of gill platelets per gram of body weight for the sixteen species of crabs, arranged according to families. In addition

TABLE I

Gill area and gill platelet number in crabs

Species	No. of determinations	Body weight grams			Platelet number			Gill area mm.²/gm.			Body vol.* Gill vol. ratio	Oxygen** µl./gm./min.
		Min.	Max.	Aver.	Max.	Min.	Aver.	Max.	Min.	Aver.		
Ocypodidae:												
Ocypode albicans	31	11.2	77.3	45.8	93	13	31	446	197	325	67.4	2.35
Uca minax	33	3.8	11.8	6.9	238	74	131	904	282	513	40.0	1.28
♂	21	4.1	11.8	7.9	199	74	115	701	282	482	45.9	
♀	12	3.8	6.7	5.1	238	117	159	904	402	567	34.1	
Uca pugnax ♂	5	1.0	2.5	2.1	739	306	487	889	658	770	57.1	
Uca pugilator ♂	7	1.6	3.0	2.3	431	241	321	817	455	624	60.3	2.03
Grapsidae:												
Sesarma cinerea	13	0.9	2.0	1.5	1178	567	830	874	480	638	63.3	2.21
Sesarma reticulata	8	7.1	11.0	8.9	208	138	183	749	493	579		
Xanthidae:												
Panopeus herbstii	38	3.3	43.0	19.2	430	27	135	1561	543	874	35.8	0.93
Menippe mercenaria	55	14.3	646.1	162.7	205	10	56	1532	386	887	33.7	0.51
Inchidae:												
Libinia dubia	12	14.8	392.0	147.2	215	10	48	1355	441	748	27.6	0.42
Libinia emarginata	26	32.3	640.8	194.9	80	6	31	1007	377	566		
♂	15	82.3	640.8	299.2	35	6	16	577	377	481		
♀	11	32.3	83.5	52.7	80	35	51	1007	535	682		
Callipidae:												
Hepatus epheliticus	6	12.1	91.8	44.3	198	28	84	1486	729	1099		
Portunidae:												
Portunus spinimanus	9	8.5	60.5	29.8	355	82	170	1107	816	901		
Portunus gibesii	5	6.2	15.3	10.3	462	212	321	1214	831	1003		
Ovalipes o. ocellatus	6	15.1	19.5	17.7	225	181	192	1512	1079	1288		
Areneus cribarus	3	70.0	220.2	122.0	65	25	53	1582	957	1301		
Callinectes sapidus	38	12.8	309.8	142.5	336	14	62	2038	734	1367	22.7	1.14

* From Pearse (1929a).
** From Vernberg (1956).

to the averages it is necessary to show the ranges of platelet number and gill area for, as will be shown, these vary inversely as the weight changes. Included also are the body volume-gill volume ratios of Pearse (1929a) for some of the same species, and, as an indication of metabolic activity, data on oxygen consumption from Vernberg (1956). Species averages of gill area within each family are relatively close, but family averages in some cases differ widely.

Pearse (1929a, 1929b) has shown that there is a tendency toward reduction in the number and volume of the gills as crabs emigrate from ocean over the beaches to land. There are sixteen gills in the wholly aquatic portunid crabs, compared to only

twelve in the Ocypodidae. Pearse also found that the ratio of body volume to gill volume varied from 22.7 in aquatic *Callinectes* to 64.7 in land-living *Ocypode*, with the more transitional species somewhere in between. Pearse did not determine gill area, which is more significant than gill volume. While it may apply in general it cannot be safely assumed that gill volume is necessarily in all cases an indication of gill area. The blue crab, *Callinectes*, and the spider crab, *Libinia dubia*, according to Pearse, have quite similar body volume-gill volume ratios (22.7 and 27.6, respectively) and for this reason might be expected to have similar gill areas, yet, as may be seen in Table I, the gill area of the blue crab is nearly double that of the spider crab.

Nevertheless, as is evident from Table II where the crabs, with their average gill areas, are arranged according to habitat, there is adequate support for Pearse's contention. There does appear to be a tendency toward reduction of gill area in those that spend part of their time on land compared to the strictly aquatic species. An aquatic crab with less gill area than an intertidal or land crab undoubtedly has lower metabolic activity. Obviously habitat is important in determining the needs

TABLE II

Crabs, with their average gill areas per gram, arranged by habitat

Aquatic		Low tide		Intertidal		Above tide	
Callinectes	1367	*Menippe*	887	*U. pugnax*	770	*S. cinerea*	638
Areneus	1301	*Panopeus*	874	*U. pugilator*	624	*Ocypode*	325
Ovalipes	1288			*S. reticulata*	579		
Hepatus	1099			*U. minax*	513		
P. gibesii	1003						
P. spinimanus	901						
L. dubia	748						
L. emarginata	566						

of respiratory surface. With an abundant oxygen supply crabs living part of the time in air do not need as much respiratory area as those living wholly in water, provided they can keep their gills moist. It is suggested that to prevent desiccation is the reason the ghost crab and the wharf crab are mainly nocturnal in the summer months. Both species have been known to succumb within fifteen minutes when confined on hot dry sand.

Habitat alone, however, does not account for the differences in gill area; rate of metabolism is also important. The portunids and the land crabs, *Ocypode* and *S. cinerea*, are very active and have high rates of metabolism. *Ocypode* has reduced gill area but at the same time has developed accessory respiratory structures for breathing in air. Ayers (1938; p. 526), comparing *Libinia, Menippe, Callinectes, Panopeus, Uca* and *Ocypode*, showed that in this series of crabs at Beaufort "there is an increase in O_2 consumption as the habitat approaches land and coincident with this there is an increase in activity of the crabs." While this holds true in a general way, Ayers' own figures show exceptions, for *Callinectes*, an aquatic species, has higher oxygen consumption and is far more active than the intertidal species. Probably the metabolic activity of *Callinectes* is indicative of that of the other

portunids. Also, judging by its gill area, *Hepatus*, a very active benthic species, probably has a metabolic rate equal to or above that of the intertidal species. *Libinia* is recognized as the most sluggish of the crabs studied by Pearse (1929a), by Ayers (1938), by Vernberg (1956), and in the present discussion. Its low oxygen consumption, as reported by both Ayers and Vernberg, is in keeping with its low gill area. Although the intertidal and land crabs do not need as great respiratory surface, even though their metabolism is higher, as do the wholly aquatic species, aquatic species with high metabolism, such as the portunids, need greater gill area than do aquatic species with low metabolism, like the spider crabs.

A question may be appropriately raised at this point. If it is an adaptation for an intertidal species to have reduced gill area for breathing in air, how does the crab

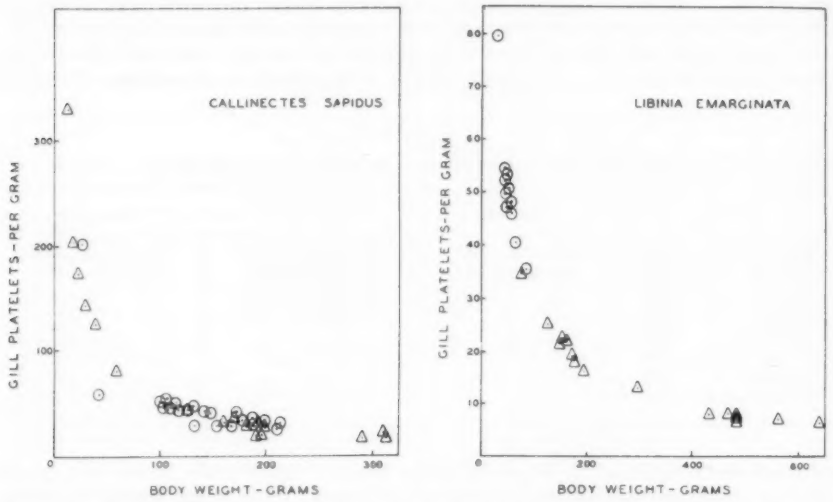

FIGURE 1. Relationship between the number of gill platelets and body weight in *Callinectes sapidus* and *Libinia emarginata*. Circles indicate females, triangles males.

survive when it is submerged? The fact that it does survive is an indication that the gill area is adequate and suggests that the crabs not only have enough gill surface for their normal needs, but enough for emergencies, too. Also, it has been demonstrated (unpublished data) that the crabs, *Callinectes* and *Panopeus* at least, go into a state of suspended animation for several hours when the O_2 tension is greatly reduced. This is long enough to carry them through a good part of a tidal cycle. In this connection, if one may be permitted to guess without supporting experimental evidence, it would be to predict that the oxygen consumption of fiddler crabs, while in their burrows with the tide covering them, is very low.

Presenting the gill areas of the various crabs as average amounts per gram of body weight suggests that the gill area per unit of measurement is relatively constant throughout life. This is not the case, however. Putter (1909) determined

the gill areas of a few crabs and fishes and maintained that the young had proportionately greater gill surface than did older animals. Krogh (1941) stated that while this was undoubtedly true Putter's work did not prove it. Figure 1, showing in *Callinectes* and *Libinia* how the number of gill *lamellae* per gram varies inversely with the weight of the crabs, and Figure 2, showing the decrease in gill area per gram of body weight with increased body growth in *Menippe* and *Libinia*, appear to substantiate the claim of Putter. Gill platelet number per gram, relatively high in very young crabs, falls off rapidly as the crabs grow older. The curves tend to level off in the older crabs. Evidently the addition of new platelets does not keep pace with the growth of the crab. Gill area per unit of weight, though more difficult to demonstrate as clearly, follows a somewhat similar pattern. Very young crabs have a greater gill surface per unit of weight than do the adults. Five small

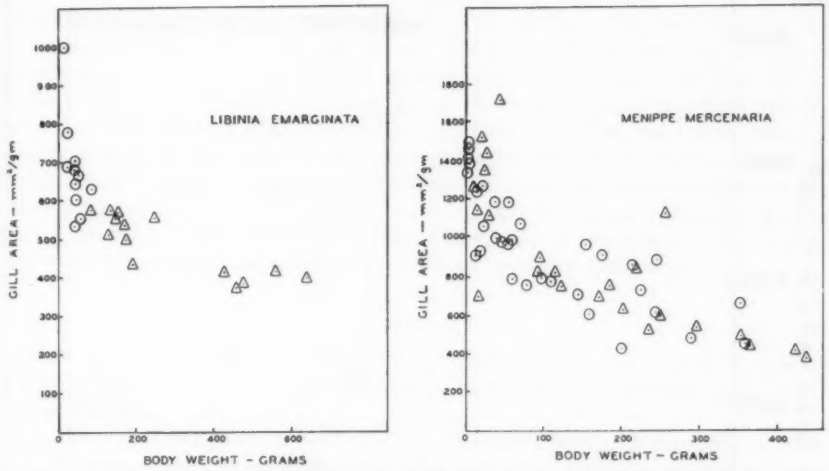

FIGURE 2. Relationship between gill area and body weight in *Libinia emarginata* and *Menippe mercenaria*. Circles indicate females, triangles males.

specimens of the genus *Menippe*, not included in Table I, weighing between 1.5 and 2.8 grams, had an average gill area of 1443 (range 1349–1496) sq. mm. per gram of body weight, whereas the gill area of individuals of the genus *Menippe* varying in weight from 10 to 600 grams averaged only about half this amount. Similarly, two crabs of the genus *Ocypode* weighing 1.0 and 2.5 grams had gill areas of 713 and 472 sq. mm. per gram of body weight, both values greater than the maximum for 31 crabs varying between 10 and 77 grams which averaged but 325 sq. mm. per gram. Though still apparent, the falling off of gill area per unit of weight is not as pronounced in older crabs as they increase in weight as it is in the young. The relative decrease in gill area is more easily demonstrated in large species that have great differences in weight between young and old than in the small species where individual variations may obscure the pattern.

It is quite possible that had Pearse (1929a) made enough determinations he

would have found that the body volume-gill volume ratio was not uniform for crabs of all sizes within the same species.

A factor deserving of comment is the percentage of inert skeleton. This differs among various species but is least in the fast-moving active land crabs, *Ocypode* and

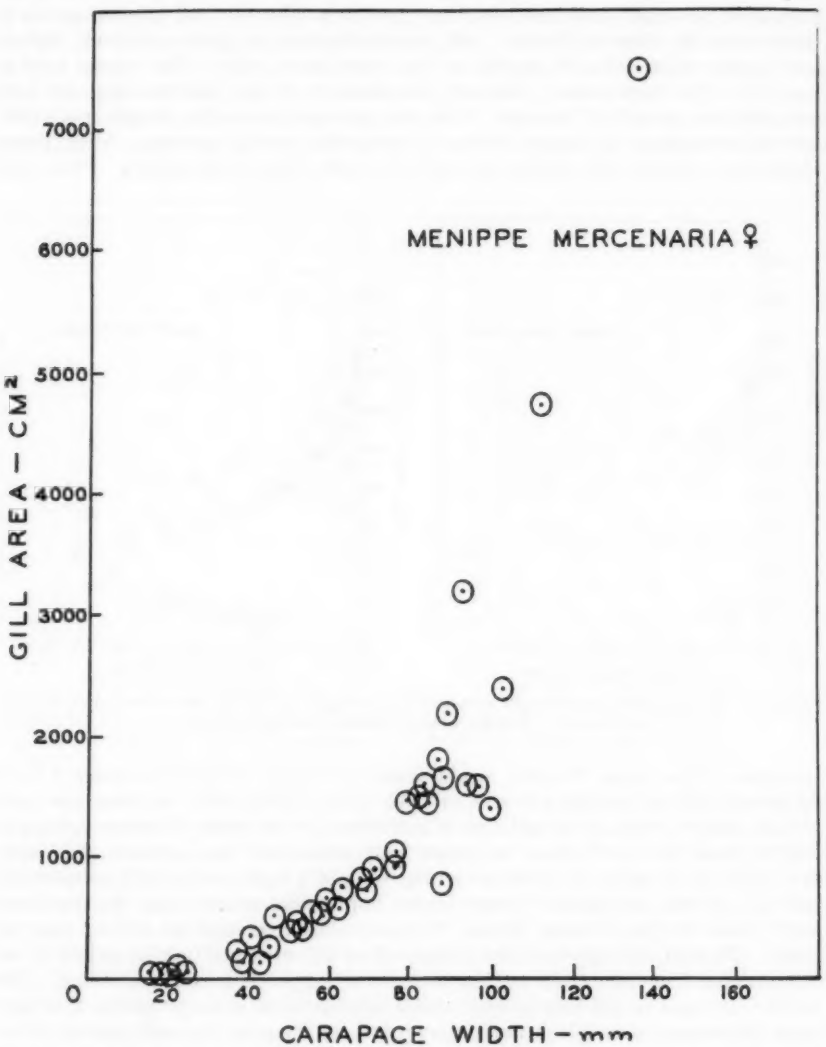

FIGURE 3. Correlation between the growth of the carapace and increase in gill area in *Menippe mercenaria*.

S. cinerea, and greatest among the heavier bodied, slower moving crabs, *Menippe* and *Panopeus.* Among the aquatic species the exoskeleton of *Callinectes* accounts for approximately 16 per cent of the total weight and that of *Libinia* about 22 per cent (unpublished data). The differences in skeletal weight, however, do not alone account for the differences in gill areas among the different species.

It may be argued that weight is not a satisfactory basis for comparing gill areas of crabs. This perhaps is true, but seems much more adequate than either body surface area or linear measurements which vary so greatly in different species. Within a species, however, linear measurements may be directly correlated with gill area. This is demonstrated in Figure 3, which shows the normal increase in total gill area of *Menippe* as the carapace increases in width.

There appears to be little or no sexual dimorphism among crabs as far as gill area or platelet number is concerned, except in those species where a major skeletal difference exists between males and females. In fiddler crabs, as illustrated by *Uca minax* (Table I), males, with greater weight because of the large chela not possessed by females, have smaller gill areas per unit of body weight than do females. In the spider crab, *Libinia emarginata,* males attain much larger size than females and may weigh several times as much. Per gram of weight the females have a greater number of gill platelets and larger gill area than do the males. It seems obvious from Figures 1 and 2 that these differences between males and females are not so much a matter of sex as of body weight. It has been found with other species, as well as with *Libinia,* that the relative number of gill platelets and the relative size of the gill surfaces decrease as the crabs grow larger and heavier.

Summary

1. A comparative study has been made of the size of the gill areas of 16 species of brachyuran crabs from six families and representing land, intertidal, and wholly aquatic habitats.

2. The size of the gill area is correlated with both habitat and metabolic activity.

3. There is a tendency toward reduction in gill area per unit of weight in going from wholly aquatic to intertidal to land species.

4. Among wholly aquatic species the active, fast moving crabs (portunids) have greater gill area than do the sluggish bottom-dwelling species (Libinia).

5. Both the gill area and the number of gill platelets per unit of weight, relatively high in very young crabs, decrease as the crabs grow older.

6. Apparent sexual dimorphism in gill area is a function of weight differences between the sexes.

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AN ANALYSIS OF RESPONSE TO OSMOTIC STRESS IN SELECTED DECAPOD CRUSTACEA

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Krogh (1939) and Prosser *et al.* (1950) have reviewed the subject of osmotic behavior in aquatic animals. Beadle (1943) has reviewed the importance of osmotic regulation in the evolutionary migration of marine animals to fresh water habitats. Pantin (1931) has discussed the origin of body fluids in animals. Robertson (1949, 1953) presents extensive information concerning ionic regulation among several groups of invertebrates. Höber *et al.* (1945) consider the physical chemistry involved in osmotic regulation. Jones (1941) showed that the crab *Pachygrapsus crassipes* regulates in dilute or concentrated sea water after 72 hours of immersion. However, osmotic regulation as a function of time for *Pachygrapsus* apparently has not been studied. This would seem to be an essential parameter in its ecologic importance, especially in the first few hours.

Salt and water pools have been suggested several times (Hukuda, 1932; Scholles, 1933; Beadle and Shaw, 1950; Gross, 1954.) The present investigation demonstrates that in the crabs studied, osmotic changes in the blood are brought about mainly by salt exchanges and not water. The presence of functional salt and water pools is considered.

Exoskeleton permeability is very unequal among decapods. Nagel (1934) found a correlation between regulating ability and permeability of the exoskeleton to applied sodium iodide in several crabs. The correlation is also established in the present study on the permeability for electrolytes and water, comparing six species of crabs and a crayfish.

The gills as seats of salt and water exchange and organs of regulation in crabs have been implicated mainly by eliminating other probable structures (Margaria, 1931; Nagel, 1934; Krogh, 1938). Webb (1940) produced histological evidence of a correlation in crabs between the ability to regulate and the complexity of the gills. Pieh (1936) demonstrated that isolated gills of regulating crabs show increased respiratory rates when exposed to osmotic stresses, thus suggesting increased work for regulation in these tissues. Koch *et al.* (1954) have produced direct evidence that *in vitro* the gills of *Eriocheir* can remove salts from a medium against a gradient. The work presented here offers direct evidence that the gill chamber of *Pachygrapsus* is a locus of electrolyte and water exchange, and that an osmotic gradient can be held in the chamber during an osmotic stress.

The energy expended for osmotic regulation has repeatedly been a subject of study by oxygen uptake determination. Schlieper (1929), Schwabe (1933) and Flemister and Flemister (1951) demonstrated that the metabolic rate increases when crabs are under osmotic stress. This they attributed to added osmotic work.

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Krogh (1939), Wikgren (1953) and Potts (1954) throw doubt on this interpretation.

The present study indicates that rates of oxygen consumption do not manifest increased osmotic work, but muscular activity.

Materials and Methods

The principal subjects for this investigation were three species of decapod Crustacea: (1) *Birgu latro* Linnaeus, the anomuran coconut crab, native of the Indo-Pacific region and an inhabitant of land, was collected on the island of Guam and maintained in the laboratory in Los Angeles. (2) *Emerita analoga* Rathbun, the common anomuran sand crab, found on sandy beaches burrowed in the sand near the level of the washing waves, was collected at Santa Monica and Corona Del Mar, California. (3) *Pachygrapsus crassipes* Randall, the brachyuran shore crab, found in high intertidal zones and in semi-terrestrial situations, was collected at Ballona Creek and Flat Rock Point, California.

The concentration of fluids was determined in two manners: (a) melting point method as described by Gross (1954), which permitted determinations on volumes as small as one $mm.^3$; (b) conductivity measurements using a 1000-cycle bridge. This allowed determinations on a two-ml. sample which was not necessarily expended but could be returned to the experimental vessel. Of course, this method measured only electrolytes. Units of resistance were converted to per cent of a standard sea water.

Oxygen consumption was measured by means of the Scholander-Wennesland respirometer as described in Wennesland (1951). All determinations were made at 16° C., a temperature to which the experimental animals were accustomed. Particular care was taken to assure that immersed animals were completely covered. No readings were made until the animal remained in the chamber for at least one hour; this was to allow them to become accustomed to the new environment.

Results

1. Osmotic Regulation as a Function of Time

Measurements of blood concentration during immersion in water were made throughout the range of water salinity, in which life could be sustained. The behavior of a species was determined partially from single readings on specimens exposed to certain stresses for given periods. However, regulation as a time function in individual specimens was followed over extended periods by two methods: (1) melting point determination on blood samples extracted periodically during immersion, and (2) periodic measurements of losses or gains of conductivity of the medium. A combination of these two methods was found best for studying individual responses over extended periods.

Emerita, a non-regulator

It was first established by melting point determinations that the body fluids of *Emerita* are isotonic to normal sea water (3.46% salt) and that this animal cannot sustain an osmotic gradient between its blood and external medium as a steady-

state condition. When three specimens were immersed in each of the following concentrations of sea water: 50, 75, 90, 110, 125, and 150% (total of 18 animals), their body fluids were isotonic to their respective external media within two hours or less after immersion. Thus *Emerita* shows no ability to regulate osmotically.

TABLE I

Solute space calculated from the relationship between concentration changes in the medium and the blood of the animal immersed in 5 times its volume of water

Specimen number	Medium (% sea water)	Change in blood (% sea water)	Change in medium (% sea water)	Change in blood / Change in medium	Calculated solute space (water equivalent) % body weight
			Emerita		
1	50	46.3	3.7	12.5	36
2	50	45.6	4.4	10.4	44
3	60	36.5	3.5	10.4	44
4	75	23.0	2.0	11.5	40
5	75	23.1	1.9	12.2	37
6	75	22.8	2.2	10.4	44
7	125	23.2	1.8	12.9	38
8	125	23.1	1.9	12.2	37
9	150	45.3	4.7	9.6	47
10	150	46.3	3.7	12.5	36
Mean				11.5	40
			Pachygrapsus		
1	25	22.6	2.7	8.4	50
1	25	15.3	1.9	8.1	51
2	50	13.7	2.0	6.9	60
3	39	13.2	1.8	7.3	57
4	50	14.7	1.9	7.8	53
5	50	14.2	1.8	7.9	53
6	50	9.5	1.3	7.3	57
7	125	10.5	1.4	7.5	56
8	145	7.9	1.0	7.9	53
9	150	15.8	2.0	7.9	53
Mean				7.7*	54

* $S = \frac{5/7.7}{1.2} \times 100 = 54\%$.

Individual specimens which had never been removed from normal sea water, were then immersed in dilute or concentrated media of 5 times the volume of the animal. Then the electrical resistance changes in the medium were followed until the resistance was stabilized and here the body fluids could be considered to be isotonic to the medium. The change in the blood concentration is therefore equal to the difference between the final concentration of the stress medium and the concentration of normal sea water. If, then, a constant ratio between osmotic changes in the blood and medium could be established, a conductivity variation in the medium

could be interpreted in terms of a change in the blood concentration, so that at any time the osmotic pressure of the blood could be estimated by such conductivity readings. Data in Table I demonstrate that such a ratio is relatively constant over a wide range of osmotic stresses, the mean value showing a change in the body fluids equivalent to 11.5% sea water for each 1% sea water change in the medium.

The rate of approaching equilibrium with the external environment suggests a physical, diffusion phenomenon. The curves are indicated in Figure 3. However, there are individual variations which result particularly with size, the smaller animals reaching equilibrium first.

Exploratory experiments suggested that *Callianassa affinis*, *Upogebia* sp., *Cancer antennarius*, *C. gracilis*, and *Pugettia producta* behave similarly.

Regulating forms immersed in water

Pachygrapsus in normal sea water is not necessarily isotonic to the medium. (Note the variation in initial blood concentration in Figure 1.) Specimens were immersed in stress media of five times their respective volumes and salinity changes in the medium were repeatedly noted by the conductivity bridge. After a significant change in the medium was observed, a melting point determination was made on the blood.

As with *Emerita*, a relatively constant ratio between conductivity change of the external medium and osmotic pressure change in the body fluids of the animal was demonstrated (Table I). On the average, a change in the external medium equivalent to 1% sea water meant a change in the blood equivalent to 7.7% sea water.

Therefore, if the initial or final blood concentration of *Pachygrapsus* were known, conductivity measurements of the medium could be converted to represent the approximate osmotic pressure of the blood.

The crabs were able to tolerate the small volumes of medium for long periods, if moved to a fresh medium of the same conductivity at least every three hours.

Over extended periods the rate of regulation in an individual specimen of *Pachygrapsus* could be estimated by converting changes in the medium to body fluid concentrations assuming the above mean ratio of change in blood to change in medium. Occasional checks were made by melting point determinations of the blood. Subsequent deviations did not exceed 10%. Such an error could not obscure a trend. When readings were not needed at close intervals, the specimen was placed in a large volume of the desired salinity and after an appropriate period a melting point determination was made on the blood. This was done on most of the late readings (*e.g.*, 72 hours). Figure 1 illustrates osmotic regulation in *Pachygrapsus* as a function of time. It should be pointed out that a small error is introduced by using the conductivity method, for when a change occurs in the medium, the osmotic gradient is consequently reduced. In the extreme case where a change in the blood was equivalent to 40% sea water, about a 5% error was effected. However, since the conductivity method was used for brief periods, not exceeding 12 hours, osmotic changes detected were small and the consequent errors caused by reducing the osmotic gradient were usually insignificant.

As shown in Figure 1, *Pachygrapsus* can regulate osmotically in both hypotonic and hypertonic media. This confirms the work of Jones (1941). However, while Jones showed this to be true after 72 hours, the present investigation shows that

regulation is established immediately, and is sustained perfectly by some specimens in moderate stresses for a few hours. Regulation then diminishes gradually until equilibrium is reached, usually within 24 hours. However, several plateaus and steps on the osmotic behavior curves may be produced before equilibrium is finally reached. Equilibrium in the case of *Pachygrapsus* does not mean that the body fluids are isotonic to the external medium. Rather, it means that the blood of the animal has reached a steady state with respect to osmotic pressure.

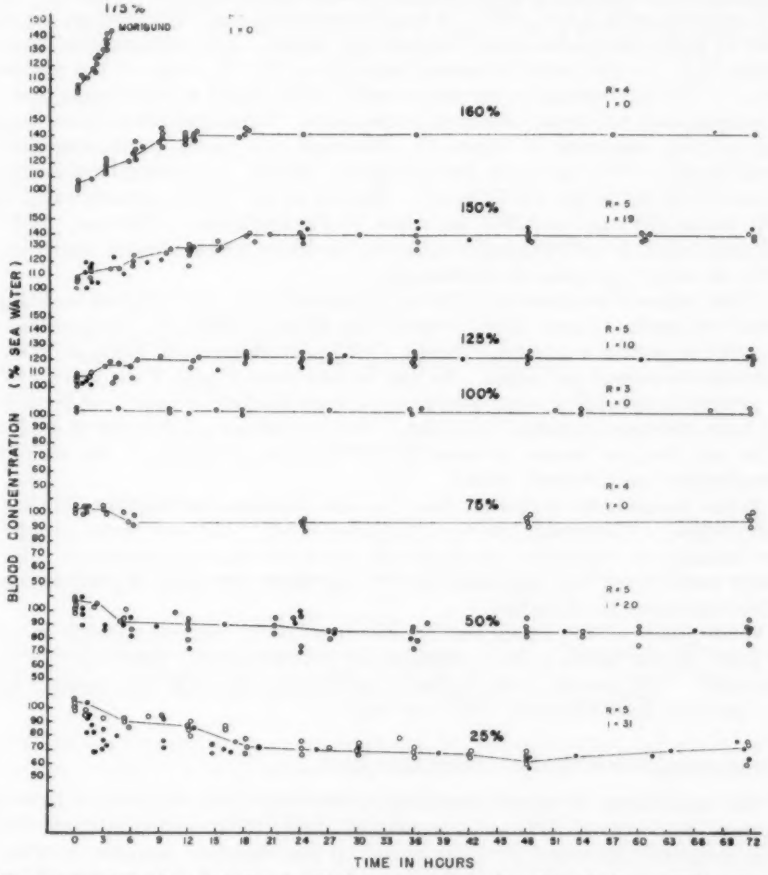

FIGURE 1. Osmotic regulation in *Pachygrapsus* as a function of time. "R" represents those specimens whose behavior was followed for extended periods by repeated determinations and is indicated by open points (○); "I" represents those specimens on which only one determination was made and is indicated by solid points (●). Solid line represents approximate median and is drawn through points representing the actual behavior of one specimen. All points are indicated unless coinciding with others. Concentration of the medium is indicated in per cent of normal sea water above the respective curve.

The plateaus and steps on the osmotic behavior curves reflect grading of the active regulatory processes or changes in accessory processes, *e.g.*, water movement over the gills. Only occasionally do fluctuations or dips occur. Such behavior is shown in Figure 1 by a crab immersed in 25% sea water.

A high degree of individual difference is demonstrated by the wide spread of points and the varying slopes among individual histories. Such variations could be caused by differences in size, age, sex, metabolic rate variances caused by physiological periodicities, *e.g.*, molting cycle, or external environment changes.

Concurring with Jones (1941), it was demonstrated that *Pachygrapsus* regulates better in hypotonic media than in hypertonic media. This phenomenon becomes evident from (a) the period of perfect regulation, (b) the slope of the regulation curve, (c) the total change in the concentration of the blood at equilibrium and (d) the extreme osmotic stresses endured by the crabs. These lines of evidence become apparent with inspection of Figure 1. However, it is worth mentioning that no animal lived in 175% sea water for as long as 6 hours. Yet several crabs actively survived 25% sea water for 72 hours. Prosser *et al.* (1955) demonstrated relatively strong tolerance to 170% sea water by *Pachygrapsus*. However, their experimental animals were gradually acclimated to lesser stresses before immersion in 170% sea water (personal communication).

There seems to be variation in the blood concentration of *Pachygrapsus* living in normal sea water. Jones (1941) reports that the body fluids of *Pachygrapsus* are hypotonic to normal sea water. Pearse (1931) found the body fluids of this crab hypertonic to normal sea water. As can be seen from Figure 1 the crabs used in the present investigation were usually hypertonic to their external medium when they were immersed in normal sea water. Such variations possibly can be explained by the fact that the osmotic pressure of the blood is a function of the molt cycle (Baumberger and Olmsted, 1928).

Token experiments suggested that *Uca* and *Hemigrapsus* regulate similarly to *Pachygrapsus*. Confirming the work of Jones (1941), *Uca* was found to regulate more strongly in hypertonic and hypotonic sea water than *Pachygrapsus*. In the salinity ranges from 50% sea water to 150% sea water, this form maintained almost perfect regulation for 36 hours.

While Jones (1941) found that *Hemigrapsus* could regulate strongly in dilute sea water, he was unable to show regulation in concentrated sea water after 72 hours immersion. The present investigation revealed that this crab can regulate up to 33% perfectly for 20 hours in 150% sea water.

Osmotic regulation in the land crab Birgus latro

The implications of osmotic regulation in the land crab *Birgus latro* have been discussed by Gross (1955). As mentioned there, these anomurans will drown when completely immersed for a day or so. It was therefore necessary to allow all the animals to rise slightly out of the water for exposure of their respiratory membranes to air. By this operation they partially released themselves from the imposed osmotic stresses. However, most of the external surface was immersed all of the time. Because of the limited number of specimens, only one specimen of *Birgus* could be studied in the representative stresses which were: 25, 50, 66 and 137% sea water, respectively (four specimens). In these cases the changes in the

osmotic pressure of the blood were followed by repeated melting point determinations on blood samples extracted at chosen times. Results are illustrated in Figure 2. These data show at least that *Birgus* is a strong regulator in both dilute and concentrated sea water. The moribund condition of the crabs after prolonged immersion in 25% and 137% sea water is not believed to be the direct result of the osmotic changes, since the two blood concentrations, specifically 70 and 119% sea water, are readily tolerated by this species (Gross, 1955). Anoxia seems to be a more satisfactory explanation for the moribund condition of these two specimens; however, high oxygen tensions failed to revive them. It is perhaps pertinent that

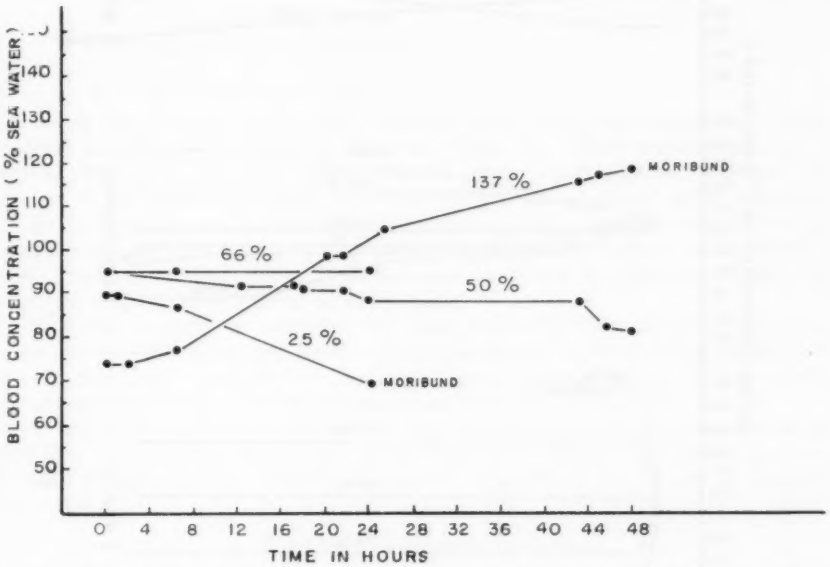

FIGURE 2. Osmotic regulation in *Birgus* as a function of time. Behavior of four specimens demonstrated by repeated melting point determinations on the blood. Concentrations of the medium are indicated in per cent of normal sea water over the respective curves.

the two animals exposed to the greatest stresses were most affected, but the fact that *Birgus* can regulate in concentrated sea water corroborates the observation that hypo-osmotic regulation is common among crabs showing some degree of terrestrial behavior (Jones, 1941; Gross, 1955).

Salt exchanges

When *Emerita* and *Pachygrapsus* are immersed in dilute or concentrated sea water, the osmotic pressure of their body fluids changes but corresponding weight changes under these conditions are small. In the case of *Emerita* a change in the body fluids equivalent to 25% sea water resulted in a weight change of less than 2% of the body weight. If pure water, this could effect a concentration change in the

blood of less than 6% on the assumption of 40% of the body weight being osmotically active water. This of course means that changes in the concentration of the body fluids are effected mostly by net changes in the solute content rather than water; 20% of the total concentration change of the blood was caused by water and 80% by solutes, in this case.

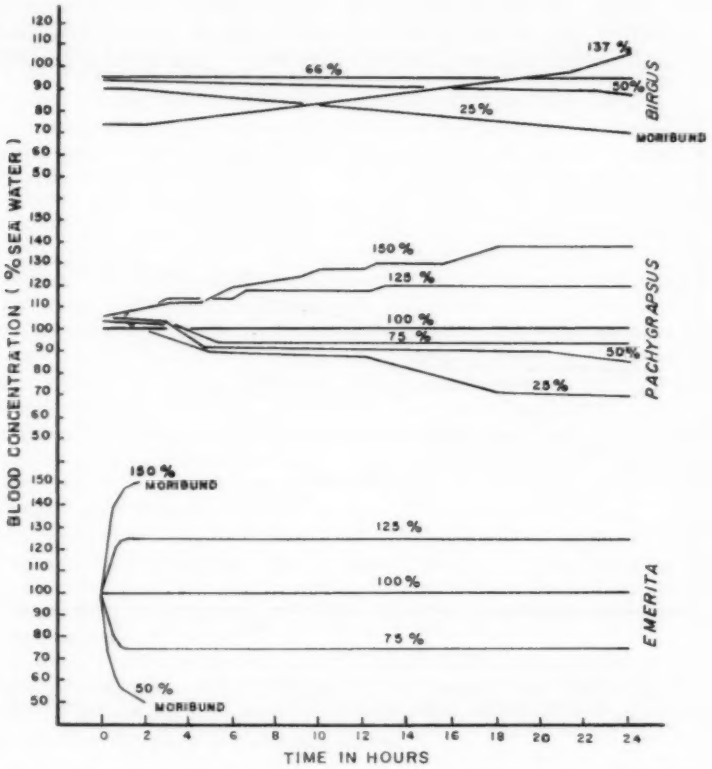

FIGURE 3. Comparative osmotic behavior of *Emerita*, *Pachygrapsus* and *Birgus*. Solid line indicates behavior of individual specimen which, in the case of *Emerita* and *Pachygrapsus*, represents the approximate average behavior of all individuals investigated for a given osmotic stress. All cases of *Birgus* are illustrated. Medium concentrations are indicated in per cent of normal sea water over the respective curves.

Curves for *Emerita* uncorrected for salt losses to the medium.

On the other hand, weight changes in *Pachygrapsus* during prolonged immersion in dilute and concentrated sea water deviating 75% from normal were so small they could hardly be considered significant. If water does cause changes in the blood concentration of these crabs, its effect is small. These findings are in accord with the work of Hukuda (1932).

Now the relatively persistent ratio between the concentration change in the blood and the external medium (Table I) suggests a method for estimating values for solute space which can be calculated from the equation:

$$S = \frac{v/p}{d} \times 100,$$

where S = solute space (equivalent in water) in per cent body weight,

$$v = \frac{\text{volume medium}}{\text{volume of specimen}},$$

$$p = \frac{\text{change in body fluids}}{\text{change in external medium}},$$

d = specific gravity of specimen.

Mean solute space values thus calculated were 54% body weight for *Pachygrapsus* and 40% body weight for *Emerita* (Table I). There was no evidence of a trend; that is, the values for solute space did not vary with different magnitudes of change in the osmotic pressure of the body fluids. Thus, if salt pools contribute to the osmo-regulatory mechanism they probably exert a constant effect over the range tested. That is, there is no varying degree of salt fixation or mobilization with increased osmotic stress, a phenomenon suggested by Hukuda (1932) and demonstrated by Gross (1954) in sipunculids.

It should be borne in mind that the above values for solute space are approximate and probably good only for the specific conditions of the experiment, for it is known that blood concentration and total water content change during certain phases of the molting cycle (Baumberger and Olmsted, 1928).

When solute space was estimated on two specimens of *Birgus*, values were 44 and 56% body weight, respectively, data which were subject to considerable error because changes in the external medium by evaporation could not be properly corrected.

2. The Osmo-Regulatory Mechanism

Jones (1941) and Prosser *et al.* (1955) have shown that the green glands of *Pachygrapsus* are ineffective as osmo-regulatory organs. Confirmatory evidence was established in the present investigation when the urine of 5 crabs immersed for 24 hours in 50% sea water was shown to be isotonic to the blood in all cases. Other regulatory mechanisms will be examined in the following section.

Osmotic regulation and permeability of the exoskeleton

Nagel (1934) demonstrated in several decapod Crustacea that non-regulators have more permeable exoskeletons than regulators. An attempt was therefore made to detect a correlation between the ability to regulate osmotically and the permeability of the exoskeleton in *Cambarus clarkii, Pachygrapsus, Hemigrapsus nudus, H. oregonensis, Cancer antennarius, C. gracilis* and *Pugettia producta.*

Since it was desired to know the role of exoskeleton in osmotic regulation of the different species, it was necessary to test the permeability of equal areas of exoskeleton of animals of about the same size. Discs of exoskeleton approximately three

cm. in diameter were removed from the carapaces of freshly killed animals. Each disc, hypodermis removed, was fitted against a rim of the end of a screw sleeve, then screwed into the end of a glass tube so that the chitinous disc formed the bottom surface (both inside and out) of the glass tube. The opening at the end of the screw sleeve determined the area of the chitin to be exposed, and was uniform for all cases. The tube was then filled with 10 ml. of normal sea water and the chitinous end was immersed in another tube containing 10 ml. of 50 per cent sea water. While the normal sea water inside the smaller tube simulated the body fluids of an animal, the dilute sea water in the larger tube simulated the external medium. The salt change in the dilute medium was then measured by a conductivity bridge after 24 hours to determine the relative permeability. As a check against leaks of water between the rim of the sleeve and disc, a few drops of concentrated dye were placed in the sea water and when such a leak occurred the dye appeared in the outside dilute medium. There was no intentional agitation and conditions for the different species were essentially uniform. Individuals chosen were apparently not close to molt.

Since the salt concentration in the dilute medium increased measurably in all cases, it can be said that the exoskeleton in all species studied is permeable either to salts or to water. If the exoskeleton were permeable only to water, then the volume of fluid in the tube which originally contained 100 per cent sea water should increase; this was never observed. The salinity changes through the exoskeleton of regulating crabs were small. However, if distilled water is used in the outside medium instead of 50 per cent sea water, a 5 per cent change can be effected in the concentration of the inner medium within 24 hours, using samples of *H. oregonensis.* This would afford a detectable volume change were semi-permeability the nature of the exoskeleton, but no volume change was observed. Thus it can be said that the exoskeleton of all non-regulators studied, and at least *Hemigrapsus oregonensis* among the regulators, is permeable to both salts and water. The average permeability of three samples of exoskeleton from each species is expressed in Figure 4. The values should be slightly higher than indicated because the osmotic gradient was reduced as salinity changes occurred. Such differences, however, would be insignificant for regulators.

It can be seen from these results that there is a correlation between regulating ability and exoskeleton permeability. The animal which probably endures the highest osmotic stress in nature, *Cambarus,* shows the lowest permeability value, although sufficient determinations were not made to state that this is significantly different from the values for *Pachygrapsus* and *H. nudus.* The non-regulator which demonstrates the lowest permeability, *C. gracilis,* still shows a value which is three times that of *H. oregonensis,* which has the highest permeability among the regulators.

Pachygrapsus is probably the best regulator among the crabs and it shows the lowest permeability value, but whether the correlation is this good cannot be said on the basis of the evidence available. Too few cases have been used to place weight on the apparent difference between *H. nudus* and *H. oregonensis.* The differences among the non-regulators do not seem to be adaptive, since these forms are stenohaline.

In order to determine whether or not the chitinous exoskeleton shows greater permeability in one direction, a section of the exoskeleton of *Pachygrapsus* was tested as above, first measuring the conductivity changes in the outer medium of

distilled water after four hours when the inner medium is normal sea water, then reversing the media, so that the sea water is on the outside, and measuring the salinity increase in the inner medium of distilled water after four hours. Permeability on three samples seemed approximately equal in both directions.

An interesting question arises as to whether regulating and non-regulating crabs differ in their active mechanisms. Could *Pachygrapsus,* for example, regulate in

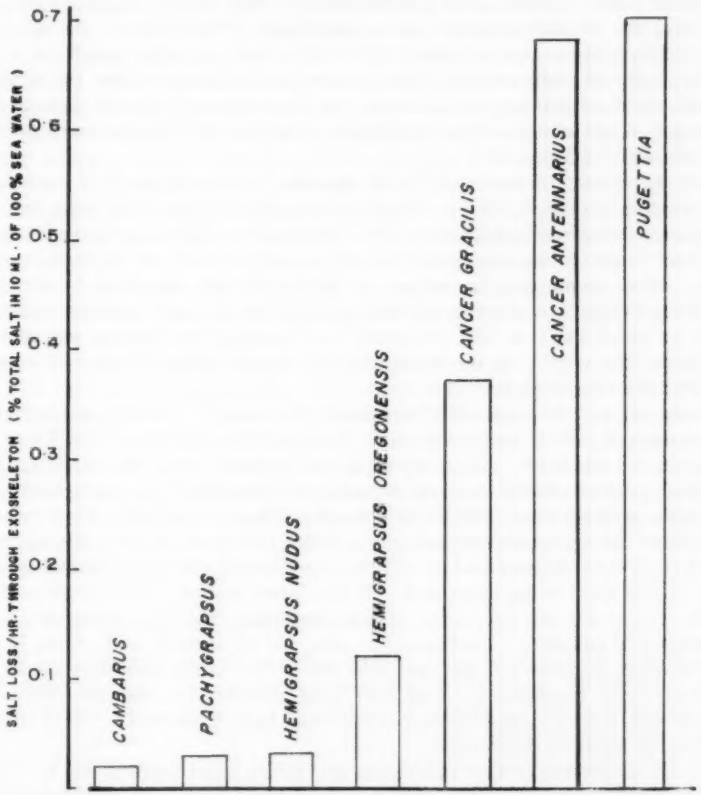

FIGURE 4. The relative permeability of exoskeleton in several decapod Crustacea. Values represent the means of three determinations for each species with a 50% sea water gradient. Variation among the three determinations for each species was in no case more than 25%.

dilute sea water if it were as permeable as *Cancer?* Much evidence is available demonstrating that animals which cannot regulate osmotically are strong ionic regulators (Prosser *et al.,* 1950). It may be that in the case of crabs, the mechanism for active, osmotic regulation is present and functioning in the non-regulators, but the permeability of the exoskeleton is so great that an osmotic gradient cannot be sustained. No evidence has been obtained on this point with respect to crabs but it

has been shown in the case of poikilosmotic sipunculids that salts can be removed from the medium without benefit of a gradient (Gross, 1954).

Osmotic regulation in the gill chamber

It is generally believed that the gills of marine crabs function as osmotic regulatory organs. Koch *et al.* (1954) have produced direct evidence in *Eriocheir* supporting this view. Gross (1955) demonstrated that the gill chamber of *Pachygrapsus* is a site of salt exchange under conditions of desiccation. As one way of testing whether this is more generally true, the salinity changes which occur in the branchial cavity of crabs removed from water were measured after the cavity was filled with water of varying salt content. It was previously shown that no significant changes occur under similar conditions when the gill chambers are filled with normal sea water (Gross, 1955).

First, the volume of fluid held in the chambers was estimated by a method reported previously (Gross, 1955). Crabs whose gill chambers had been pierced for flushing were immersed in tap water, 25% sea water or 150% sea water for a period of one hour; each cavity was then flushed out with 10 ml. of distilled water, the flushings were caught and the salinity of this fluid was measured by means of a conductivity bridge. Assuming that the gill fluid is the same concentration as the medium in which the crab was immersed and knowing the volume and salinity of the flushings, the volume of the branchial fluid can be estimated as well as the absolute quantity of salt in that fluid.

The animal was then immersed in normal sea water for about two hours to permit recovery. Again it was returned to its respective stress medium for an additional period of one hour. The crab then was removed from the water and placed in a closed container which was kept at saturated humidity by a paper towel soaked in the same medium from which the animal had been removed. Such paper was placed where the crab could not reach it. After two to seven days the animal was removed from the container and its gill chamber flushed out with distilled water, the salinity of this fluid being measured. If the latter salinity determination differed from the former for the same crab, it was suggested that salt exchanges occurred in the branchial chamber. Such was the case, for all animals with dilute branchial fluid showed an absolute salt increase after the period in the humid container, while all animals with concentrated branchial fluid showed an absolute decrease. It should be said, however, that the crabs which had been immersed in 150% sea water showed some signs of desiccation.

It is not surprising to find salt exchanges in the gill chamber, for as described above, even the chitinous exoskeleton is permeable to both salts and water. However, if it could be demonstrated that the salinity of the branchial fluid were different from that of the blood when a steady-state had been reached, then it would seem that a dynamic mechanism is present in the gill chamber, permitting an exchange of salt with the body fluids, but also being able to hold an osmotic gradient.

Now, the volume of branchial fluid for each animal was determined with the first flushing operation described above. Thus, the salinity of the second flushing will yield an estimation of the branchial fluid concentration after the period in the humid container. Results suggest that a gradient is sustained in the gill chambers of all animals which had been immersed in dilute media. The mean salinity of such

fluid from two animals which had been immersed in tap water was equivalent to 14% sea water while the blood was estimated to be greater than 75% sea water in concentration. The mean salinity in the gill chamber of four animals removed from 25% sea water was equivalent to 64% sea water while the blood concentrations were estimated to be greater than 85% sea water. Data from the specimens which had been immersed in 150% sea water were considered invalid for this aspect of the experiment.

It might be argued that the apparent gradients described above are not real, but that a great amount of branchial fluid rests on impermeable tissues. In such a case, the fluid in contact with the permeable tissues could become isotonic with the blood and this would affect the total salt content in the gill chamber only enough to give the impression that an osmotic gradient was being held. However, the large apparent gradient sustained when the branchial fluid was initially tap water, and the large salt exchange under conditions at desiccation (Gross, 1955), do not support this argument. Since all animals remained out of the water for at least two days before the salinity of their branchial fluid was measured, there was plenty of opportunity for salt exchanges to take place. It is probable, therefore, that the above described gradients are real, but the fact that more salt change occurred for animals from 25% sea water than for those from tap water raises doubt as to the quantitative value of the data, since the osmotic gradient between blood and gill chamber was greater for the tap water animals.

One more point of interest arises from this experiment. It was previously estimated that the mean branchial fluid volume for 20 *Pachygrapsus* was 1.7% body weight (Gross, 1955). The mean branchial fluid volume of those animals immersed in 150% sea water was only 0.71% body weight. Yet, again, those immersed in dilute sea water averaged 1.7% body weight. This, of course, suggests that *Pachygrapsus* has some control over the water that enters the gill chamber, which would be an aid to osmotic regulation. If such an aiding mechanism for regulation exists, it seems that it functions only for hyper-regulation, but confirmation is needed for this finding.

Metabolic work in increased osmotic stresses

It has been reported that when certain crabs are exposed to osmotic stresses, they respire more rapidly; this increase in metabolism has been interpreted as work accomplished by the regulatory mechanism (Schlieper, 1929; Schwabe, 1933; Flemister and Flemister, 1951). However, Krogh (1939) suggested that such metabolic increases are caused in part by activities of the organism other than osmotic regulation.

I have observed that certain crabs, *e.g., Pachygrapsus,* show violent attempts to escape from a medium which departs much from normal sea water in concentration. Obviously such activities would step up the entire metabolic rate of the crab, making it difficult to show the increased rate due to osmotic regulation alone.

The necessary increase in the osmo-regulatory mechanism cannot be predicted simply by knowing the normal osmotic pressure of the body fluids of an animal and the osmotic stress imposed. Rather, a knowledge of the sustained osmotic gradient is needed to give the relative amount of regulation necessary to withstand a certain stress. It is conceivable that a given amount of osmotic work will main-

tain the body fluids at viable concentrations over a range of osmotic stresses. This could be possible if the animal holds a constant gradient between blood and external medium, although the actual blood concentration alters to permit the constant gradient. Over such a range of stress, then, the constant rate of regulation would be

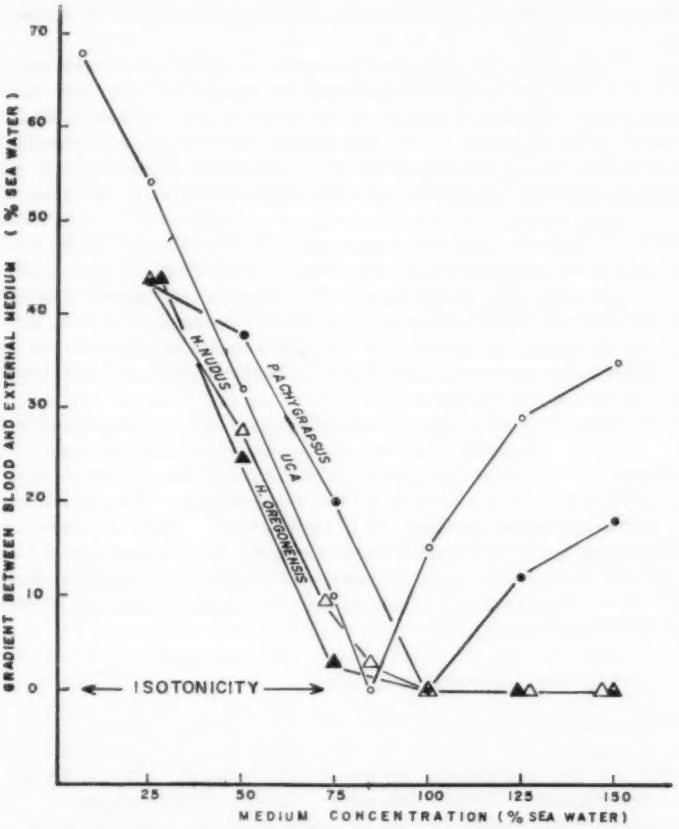

FIGURE 5. Crabs sustain greater osmotic gradients at equilibrium as greater stresses are imposed. Points are approximate mean values and for *Pachygrapsus* are calculated from the present investigation; values for *Uca*, *H. oregonensis*, and *H. nudus*, are calculated from Jones (1941).

expected to cause no variations in the total metabolic rate assuming constant relative roles of the various regulatory organs. On the other hand, if an increasing gradient is established more work would be expected. It has been pointed out recently by Potts (1954) that semi-permeable animals inhabiting fresh water could maintain their blood hypertonic to the medium with less work by excretion of dilute urine than by regulating at the tissue surfaces which are exposed to the external medium.

However, it was also demonstrated that such a mechanism would not be significantly advantageous over the other unless large osmotic gradients were sustained between the blood and external medium (greater than 50% sea water). The possibility should therefore be considered that at certain stresses the mechanism of regulation may shift from one process to another, and that the various processes are not neces-

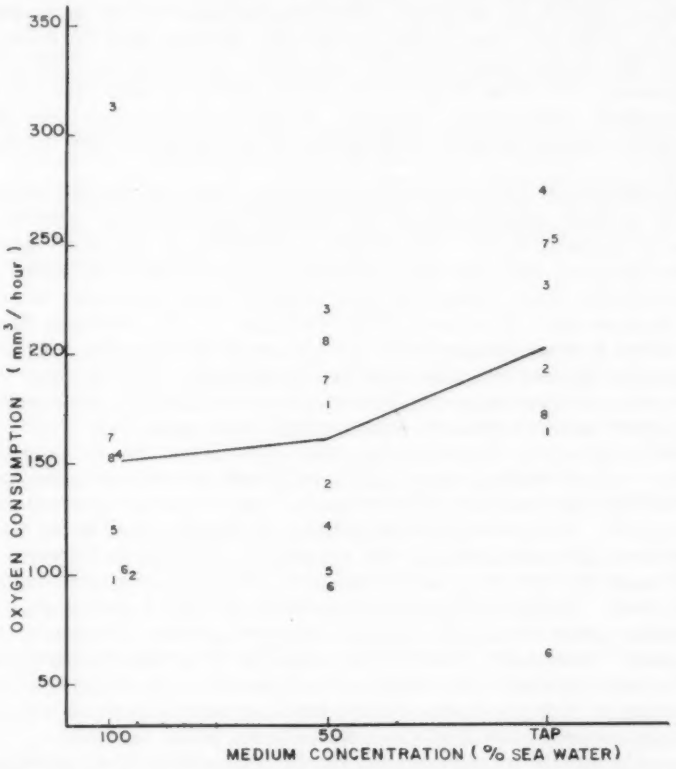

FIGURE 6. Oxygen consumption in *Uca* as a function of osmotic stress. Numerals represent individual specimens. Solid line connects mean values for all cases. Measurements made at 16° C. on crabs of approximately two grams. These could not stand out of the solution.

sarily equally efficient. Nevertheless, it does not seem to be unreasonable to assume that in general more metabolic work is required to sustain a large osmotic gradient than to sustain a small osmotic gradient.

Figure 5 demonstrates that in *Uca, Pachygrapsus, Hemigrapsus nudus,* and *H. oregonensis,* more osmotic work would be expected in greater stresses because the sustained gradients increase with the larger stresses, except for the two species of *Hemigrapsus* when they are immersed in concentrated sea water where they cannot regulate. It can be observed in *Pachygrapsus* that the sustained gradient in-

creases almost linearly with the stress, from a medium of normal sea water up to one of 50% sea water; but in 25% sea water the gradient drops off from the linear relationship with stress. This indicates that the crab must work only slightly faster, and it can be seen that with the same metabolic output an equal gradient could be held in even more dilute water. *Uca* shows almost a linear increase in the gradient up to a medium of 25% sea water and only a slight fall off from this relationship to stress in 5% sea water. *Hemigrapsus nudus* and *H. oregonensis* show behavior in dilute sea water which is somewhat different from *Pachygrapsus* and *Uca*, for they regulate weakly in 75% sea water, sustaining less than a 10% sea water gradient. With like stresses *Uca* and *Pachygrapsus* hold 22% and 20% sea water gradients, respectively. However, as the stress increases both species of *Hemigrapsus* regulate strongly, as illustrated by the increased slopes for these crabs in Figure 5.

If the additional osmotic work could manifest itself, the animals would be expected to consume more oxygen as the medium departs farther from the osmotic pressure of the body fluids. The oxygen consumption of individual specimens of *Uca* was compared when the crabs were immersed in normal sea water, 50% sea water, and in tap water. Since this species may be found commonly in normal sea water, its respiratory rate was measured first in this medium, assuming that harmful effects would be at a minimum here. For the next determination, half the animals were tested in 50% sea water, the other half in tap water. For the third determination the order was reversed so that each specimen was studied in three media. This was a control against permanent injury which might result from the heavy stress imposed by tap water. However, the order of immersion did not seem to make a difference. After determinations in 50% sea water and tap water, the crabs were placed in 100% sea water for 24 hours to allow recovery before the next determination was made. All specimens whose behavior is recorded lived in sea water for at least 48 hours after completion of the experiment. Results in Figure 6 illustrate that the metabolic rate of an individual animal does not necessarily increase with osmotic stress. In fact, of 8 animals, only numbers 2 and 7 show such a behavior. With the exception of two extreme cases, however, number 3 in normal sea water and number 6 in tap water, the respiratory rates for the group of animals seem to be higher in higher stresses. The mean of the 8 specimens shows a value for oxygen consumption in 50% sea water of about 108% of what it is in normal sea water, while in tap water it is about 135% of the value for 100% sea water.

Assuming that the normal osmotic pressure of *Uca* blood is equivalent to that of 85% sea water, there will be a 15% sea water gradient when the crab is immersed in normal sea water; in 50% sea water there will be a maximum gradient of about 35% sea water, and in tap water a maximum gradient of 85% sea water. When the animal is exposed to an osmotic stress, the amount of work should increase by the same factor as does the increased sustained gradient. When immersed in 50% sea water, regulation might increase by 35–15 or 20 units. The mean respiratory rate in 50% sea water shows 8% increase over animals immersed in normal sea water; thus, the fraction of total metabolism responsible for regulation in sea water might be calculated by simple proportion to be 6.0%. In tap water, this value is calculated to be 7.5% of the total metabolism.

These results do not agree with those of Flemister and Flemister (1951) who

studied another crab, *Ocypode*. Flemister and Flemister regarded the increased respiratory rate in hypertonic and hypotonic sea water as a manifestation of greater chloride regulation, and probably osmotic regulation. If this is the case, then about the same amount of metabolic work serves to maintain almost perfect regulation in any medium which establishes a gradient of from 25% to 50% sea water, yet in an isotonic medium, the metabolic rate is at least 20% lower. Could it be that osmotic regulation in *Ocypode* is an all-or-none process which is triggered by a salinity change in the medium? *Uca* is also a member of the family Ocypodidae, and its habitat is similar to that of *Ocypode*. It would seem questionable, that the osmo-regulatory mechanism of these closely related organisms could be essentially different.

It is this author's opinion that the interpretation of increased respiratory rates of animals subjected to osmotic stresses as a manifestation of greater osmotic work is to be questioned. This opinion is based on the inconsistent results found in individual animals in the present investigation (Fig. 6) and on the knowledge from numerous observations on a number of species that crabs are sensitive to osmotic stress and attempt to escape.

Discussion

The osmotic responses of the species studied in this investigation are not always obviously adaptive. The stenohaline forms represented by *Emerita* are presumably limited in their choice of habitat by their tissue tolerances, since they are unable to regulate. Yet, this species can tolerate salinities of from 75% sea water to 125% sea water for at least 24 hours, suggesting that the dilute waters of estuaries and the concentrated waters of tide pools might partially afford a refuge for them. It would seem to be the habits of this animal that dictate that it live near the level of the washing waves, rather than its lack of osmotic regulation.

The strong osmo-regulatory ability of *Pachygrapsus* and *Birgus* superficially seems superfluous in animals of their habits. *Pachygrapsus* generally is found in normal sea water where it does not have to regulate, or on land where it is subjected to desiccation, not direct osmotic forces. *Birgus* is found usually on dry land except for periods when it returns to the sea to reproduce. Gross (1955) discusses the significance of the ability to regulate among terrestrial and semi-terrestrial crabs, and suggests that in nature where osmotic stresses in aqueous media are rarely encountered it is of little importance as such, but appears under the artificial conditions of experimentation, *i.e.*, osmotic stresses, as a secondary manifestation of physiological processes important for life on land. It is interesting that the regulators *Pachygrapsus* and *Birgus* show large tolerances to variations in their blood concentrations.

The immediate resistance to osmotic stresses demonstrated by regulating animals may be a matter of inertia permitted by relatively impermeable exoskeletons, for the present investigation has shown a correlation between osmo-regulatory ability and integumental impermeability. The latter significantly contributes to the time factor which is important to intertidal animals which are subjected to tidal rhythms. In the case of *Hemigrapsus*, hypo-osmotic regulation for 20 hours would be adequate, because it would be rare for this animal to be exposed to the air for such a period.

Pantin (1931) has said that non-regulatory animals may posses the same mecha-

nisms permitting osmo-regulation as do regulators but to a lesser degree. It may be, in view of the above findings, that the difference between a regulator and a non-regulator could be merely a difference in permeability.

Previous investigations have produced evidence that the gills are major osmo-regulatory organs among marine crabs. The present investigation has established that a dynamic flux of salts and water occurs in the gill chamber of *Pachygrapsus*. Although no direct evidence has been produced, it is probable that the gill tissues are capable of actively transporting salts. However, the regulating mechanism may not lie entirely at a tissue level, for there is evidence suggesting that stress media can be partially excluded from the branchial chamber, therefore preventing contact with the gill tissues in large volumes.

The green glands of *Pachygrapsus* are probably not important organs of osmo-regulation, because the urine is close to the concentration of the blood in various concentrations of sea water. Prosser *et al.* (1955), however, have produced evidence that the green glands are important ion regulators. Potts (1954) has shown theoretically that a semi-permeable animal would not gain much regulatory efficiency in excreting a dilute urine, unless extreme stresses were encountered. However, the crabs used in this study are not semi-permeable. In fact, net changes in the osmotic pressure of the blood were shown to be effected mainly by salts, not water.

The relatively constant values for solute space which were calculated from the ratio of concentration changes in the blood to concentration changes in the external medium have interesting implications, namely, that if salt pools are contributing to the blood changes occurring under osmotic stress, they are doing so in a constant manner. The question remains open, whether or not a given alteration in the blood can be accounted for in the external medium.

When regulating crabs are exposed to increasing osmotic stresses, they generally sustain greater osmotic gradients. This would necessitate greater osmotic work. However, increased rates of respiration which are registered by crabs when they endure an osmotic stress cannot be interpreted as the manifestation of that increased work, for other activities, stimulated by the stress, *e.g.*, struggle to escape, cannot be isolated. It may be possible that greater osmotic work alone does not add appreciably to the total metabolic rate.

This investigation was conducted under the direction of Professor Theodore Holmes Bullock, to whom I am grateful for his most helpful encouragement and guidance. I am also indebted to Professor C. L. Prosser and Dr. J. D. Robertson for reading parts of the manuscript and offering helpful suggestions.

Summary

1. The decapod Crustacea, *Emerita, Callianassa, Upogebia, Cancer antennarius, C. gracilis* and *Pugettia* cannot regulate osmotically and from lack of tolerance are generally stenohaline.

2. *Pachygrapsus, Birgus, Hemigrapsus* and *Uca* can regulate osmotically in concentrated and dilute sea water. *Hemigrapsus* is the weakest hypo-osmotic regulator of the four species.

3. Among species where osmotic regulation occurs, it is established immediately and may be long lasting or may grow weaker with time. Although blood concentrations may fluctuate in a given stress, the phenomenon is not common.

4. Equilibrium of the blood concentration, where perfect regulation does not occur, is usually established within 24 hours following immersion; changes occasionally occur later when extreme stresses are imposed.

5. Estimates on the solute space volumes were calculated as 40% for *Emerita*, 54% for *Pachygrapsus* and about 50% for *Birgus*.

6. Concentration changes occurring in the blood of *Pachygrapsus* and *Emerita* are caused mostly by salt rather than water exchanges.

7. There is a dynamic flux of salt and water in the gill chamber of *Pachygrapsus*, thus furnishing further evidence that the gills are osmo-regulatory organs.

8. The osmotic regulating crustaceans *Cambarus, Pachygrapsus, Hemigrapsus nudus* and *H. oregonensis* have less permeable exoskeletons than the non-regulators, *Cancer gracilis, C. antennarius* and *Pugettia* by a factor of at least three.

9. *Pachygrapsus, Uca, H. nudus* and *H. oregonensis* sustain greater osmotic gradients when greater osmotic stresses are imposed. The two species of *Hemigrapsus* are weak regulators in small stress, but the osmo-regulatory activity accelerates as stresses increase. A sensitivity to absolute salinities is suggested.

10. *Uca* averages greater respiratory rates in greater osmotic stresses, but this is not necessarily so for individual specimens and the average differences are small. A discussion of the energetics of osmotic regulation reaches the conclusion that such increases in metabolism are not direct reflections of increased osmotic work but of muscular or other activity.

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STUDIES ON FEEDING, DIGESTION, AND FOOD STORAGE IN FREE-LIVING FLATWORMS (PLATYHELMINTHES: TURBELLARIA)

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Investigations upon turbellarian nutrition (summarized by Hyman, 1951 and Yonge, 1954) have so far dealt mainly with triclads, where intracellular digestion has been demonstrated but the possibility of some supplementary intraluminar digestion not fully explored. Triclad nutrition has therefore been re-examined, using *Polycelis cornuta*, and the opportunity taken to make comparable investigations on representatives of the other turbellarian orders. In each case the nature of the food and the feeding mechanism, the structure of the gut and the course of digestion, and the nature and location of the food reserves have been studied. The various mucoid and rhabdoid secretions have also been examined and an assessment made of their value in feeding.

Materials and Methods

The following Turbellaria, listed systematically with details of their habitat, were examined.

Order Acoela. *Convoluta paradoxa*, Oersted. From rock pools rich in *Ulva* and *Enteromorpha* in Cullercoats Bay, Northumberland.

Order Rhabdocoela. Sub-order Notandropora. *Macrostomum* sp., Oe.

Sub-order Opisthandropora. *Stenostomum* sp., Duges. Both from ponds in the Leeds area.

Sub-order Lecithophora. *Mesostoma tetragonum*, O. F. Müller. From Middlerigg Tarn, Troutbeck, Westmorland.

Order Tricladida. Sub-order Paludicola. *Polycelis cornuta*, Schmanda. From under stones in small fast-running streams in the Leeds area.

Order Polycladida. Sub-order Cotylea. *Cycloporus papillosus*, Lang. On colonial tunicates (*Botryllus* and *Botrylloides*) beneath weed-covered rocks at low-water mark on St. Mary's Island, Northumberland.

Sub-order Acotylea. *Leptoplana tremellaris*, O. F. M. Beneath stones on a predominantly sandy shore at Llanfairfechan, North Wales.

The flatworms were starved to encourage a readiness to feed and to clear the gut lumen and the gut cells of any remnants of previous meals. The animals associated with the flatworms under natural conditions were then presented to them so that the methods of capture and ingestion of the selected prey could be followed in detail. Flatworms were killed where possible in the act of feeding, Steinmann's fixative (equal parts concentrated nitric acid, distilled water and saturated mercuric chloride in 5% aqueous sodium chloride) giving the necessary instantaneous fixa-

tion, and after washing in running water the preparations were stained in borax carmine.

The natural food was used, whenever possible, to determine the site and course of digestion, but in forms with a phagocytic gastrodermis (especially the triclad) both this and the soft foods used by previous investigators (Arnold, 1909; Willier, Hyman and Rifenburgh, 1925; Kelley, 1931) disintegrated during ingestion and it could not be ascertained whether any intraluminar break-up preceded the phagocytosis and intracellular digestion, nor whether the separate constituents of the food (proteins, carbohydrates and fats) were dealt with differentially. Hence the flatworms were fed either on food which reached the gut in a visibly recognizable condition, or on homogeneous food substances whose digestion could be detected by simple chemical tests. Some of these were readily eaten, but with others the natural prey had to be used as a carrier. This particularly useful technique was applied by extracting the body contents of a normal prey with a hypodermic syringe and replacing them with the appropriate food substance. In all cases the flatworms were fixed at intervals after an observed feed, and examined histologically. All fixation (in Susa, saturated mercuric chloride or 10% formalin) was carried out at about 40° C. to prevent rupture or discharge of the gut contents. Sections cut at 10 μ were stained with Ehrlich's haematoxylin and eosin, Heidenhain's iron haematoxylin and Feulgen's stain. Squash preparations were also made, either directly in 0.5% saline or after preliminary maceration in saturated aqueous boric acid, and examined both fresh, and fixed and stained. In cases where ingested food was visible within the living flatworm its fate was also followed by direct observation.

Fat reserves were studied from squashes fixed in osmium tetroxide vapour and from sections prepared after fixation in Flemming's fluid. Carbohydrate reserves were studied after fixation in 95% alcohol, the sections being stained with Best's carmine for glycogen or periodic acid-Schiff's reagent for carbohydrates generally. For protein reserves flatworms were fixed in neutral 10% formalin and the sections stained by the modified Millon method (Bensley and Gersh, 1933).

Special methods for the study of pH changes during digestion and the examination of the mucoid and rhabdoid secretions are described in the text.

Observations

TRICLADIDA. *Polycelis cornuta*

The food and feeding mechanisms

P. cornuta (1–1.5 cm. long and 2–3 mm. broad) feeds upon small annelids, crustaceans and insect larvae, particularly those of Ephemeroptera, Plecoptera and Diptera. It is also a scavenger and feeds upon injured or dead animals, provided they are not too decomposed—juices diffusing from such animals attract all the flatworms in the vicinity and they can be collected in great numbers by anchoring a crushed earthworm in the stream as bait.

The living prey may be seized directly but is often caught after it has become entangled in the mucus produced for locomotion and adhesion by the *Polycelis* population in general. The normal slow movement by ciliary gliding is facilitated by mucus secretions which are laid down in tracts comparable to the slime trail of a snail; this mucus is not particularly sticky but small animals are occasionally trapped

in it. Often, however, ciliary locomotion is supplemented by direct muscular movement in which waves of contraction pass down the body and urge it forward. During such movement, and particularly when the flatworm is facing a current or changing levels upon a plant or stone, mucus of the type usually used for adhesion when at rest is produced from the edges of the body to provide temporary anchorage during the muscular contractions. This mucus is very sticky and as the flatworm moves it is drawn out into strands 2–3 cm. long which stretch between stones and leaves or along a level substratum. The many single strands produced in this way tend to cross or coalesce to form a complex tangle which becomes a most effective trap for small animals. Such deposits occur all over the natural habitat, and can be easily demonstrated in laboratory culture dishes by flooding with weak eosin (Fig. 11). *Polycelis* is gregarious and this habit results in a large amount of mucus being deposited within a given area. Animals with many appendages, such as crustaceans and insect larvae, are easily trapped in these mucus "snares" and become still more entangled in their struggle to escape. The flatworm does not lie in wait near the "snares" but is rapidly attracted by any disturbance in the water created by the struggles of the prey. Several individuals are often attracted to the same prey and their concerted efforts enable them to feed upon animals too large for a single individual. The prey is seized by the flatworm wrapping itself about it and covering it with large amounts of the sticky mucus. In this way even relatively large animals such as gammarids are rapidly immobilized. There is no evidence of any toxic effects of the mucus since prey rescued after complete immobilization show no ill effects. Inert food does not stimulate the formation of much mucus; only the amount necessary for adhesion is secreted when such food is being eaten. After the prey is seized the pharynx is protruded and makes exploratory movements over the body surface. With arthropods it is eventually forced through some weak point in the exoskeleton, as between sclerites or the area of articulation of a limb, and rapidly sucks out the body contents (Fig. 10). The pharynx is very extensible and moves about within the prey, entering the head and larger appendages to draw out all the organs and musculature, so that virtually only the empty exoskeleton is finally left. With annelids the cuticle is breached and the body of the worm extracted, whilst with suitable carrion minute pieces are sucked off by the pharynx. The disintegration of the food on its way to the gut is so rapid that it appears to be due entirely to mechanical disruption during its passage through the pharynx; there is no evidence that digestive juices assist the process since the waves of muscular contraction in the pharynx invariably pass backwards towards the gut, never forwards as would be expected if such juices were being regurgitated. The efficacy of the process is indicated by the homogenized condition of the food as it arrives in the gut lumen.

The structure of the gut and course of digestion

The large cylindrical plicate pharynx lies in the pharyngeal chamber in the posterior half of the body and is directed backwards so that it can be protruded through the posterior central "mouth" by simple muscular elongation (Fig. 1). It is very muscular and contains both eosinophilic and basophilic mucus glands whose secretions help ingestion.

The gastrodermis (Figs. 12 and 14) consists of a single layer of cells resting upon a delicate basement membrane. There are two types of cells, of which the

larger and more numerous are columnar, 35–40 μ in height with basal vesicular nuclei; their distal ends often bulge freely into the gut lumen and their basophilic cytoplasm is finely granular and may contain various eosinophilic inclusions. These inclusions disappear progressively after feeding and hence appear to represent phagocytosed food undergoing intracellular digestion. The second type of gut cell, later referred to as "sphere cells" on account of their appearance, is 20–30 μ high and normally contains intensely staining spheres which appear to constitute a protein reserve.

The finely divided condition and mixed nature of the normal food after ingestion made it difficult to determine whether phagocytosis was preceded by any intraluminar break-up, and experimental feeding by the carrier technique was applied.

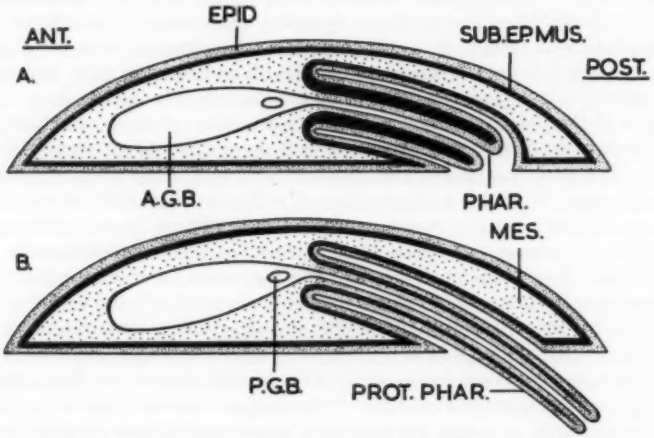


FIGURE 1. Diagrammatic longitudinal sections of *Polycelis* to show the cylindrical plicate pharynx. A, The normal condition with pharynx retracted. B, Pharynx protruded for feeding; ant.: anterior; a.g.b.: anterior gut branch; ev.phar.: everted pharynx; m.g.b.: median gut branch; mes.: mesenchyme; phar.: pharynx; post.: posterior; p.g.b.: origin of posterior gut branches; prot.phar.: protruded pharynx; sub.ep.mus.: subepidermal muscles.

The carriers used in this case were gammarids which were boiled before injection to ensure that no active enzymatic juices of the crustacean remained to attack the test food. The series of test foods was chosen with three objectives: (a) to follow the separate fates of carbohydrates, proteins and fats; (b) to supply unequivocal evidence of the presence or absence of intraluminar digestion; and (c) to determine the maximum size of particle which can be phagocytosed by the gut cells.

(1) Experimental feeding with carbohydrates

Polycelis readily ingested boiled starch paste, and sections prepared immediately after feeding and stained with Lugol's iodine showed the blue and unchanged starch lying in the gut lumen. The paste was quickly phagocytosed and twelve hours after feeding only a small amount remained in the lumen, quite unaltered and still stained blue with Lugol, indicating the absence of any intraluminar digestion of carbohy-

drates. The intracellular digestion of the phagocytosed starch was followed in saline squashes of fed flatworms. Two hours after feeding the columnar gut cells contained large amounts (Fig. 13), which stained with Lugol in various shades of blue and brown. As time passed all stained brown and then eventually disappeared as digestion was completed. Starch was also found in mesenchymal cells so that a certain amount of phagocytosed material passes back into the mesenchyme for digestion. Indicators mixed with the starch paste were unchanged in the gut lumen, giving further evidence of the absence of intraluminar digestion, but as the pH value after phagocytosis was 6.8–7, intracellular digestion of carbohydrates occurs in a neutral to slightly acid medium.

Raw starch grains were ingested and readily phagocytosed if sufficiently small, and could be seen very clearly in sections by polarized light in the columnar cells. They were unaffected by the intracellular diastatic enzymes and were eventually rejected by the cells and returned to the gut lumen. In this case, therefore, no capacity for the selection of suitable food material appears to be possessed by the gut cells themselves. On the other hand, no grains above about 30 μ were taken up and apparently this is the critical size above which phagocytosis is impossible. Later all the starch grains were expelled in the usual manner by taking in water through the pharynx and flushing out the gut contents by violent contractions of the body.

(2) Experimental feeding with proteins

Polycelis fed directly upon clotted frog and chick blood. The erythrocytes were quickly phagocytosed and could be seen in the columnar gut cells in saline squashes made thirty minutes after feeding. The number phagocytosed increased with time and six hours later almost every cell was packed full. Occasionally erythrocytes were also seen in mesenchymal cells. The erythrocytes quickly changed in appearance after phagocytosis and condensed into homogeneous deeply staining spheres which decreased in size and number as digestion and absorption progressed (Fig. 14). They showed no signs of digestion in the gut lumen prior to phagocytosis.

Chopped frog muscle stained with various indicators was fed to the flatworms by means of gammarid carriers. The particles reached the gut in recognizable pieces still showing their characteristic striations and the larger remained unchanged in size, shape and pH value until ejected from the gut some 24 hours later. This indication of the absence of intraluminar proteolysis was confirmed by feeding coagulated albumen, gelatin and fibrinogen, all of which likewise remained unchanged throughout their stay in the gut lumen. The smaller particles of chopped muscle were phagocytosed and neutral saline squashes made one hour after feeding showed many present in the columnar gut cells. Particles stained with brom-cresol green were then a clear blue in color (pH 5.2) but changed with time until six hours after feeding when they had become a clear green (pH 4.6), so that intracellular protein digestion proceeds in a distinctly acid medium. This color faded as the particles themselves became rounded and less distinct. Stained inclusions passing through the same series of color changes were occasionally found in mesenchymal cells. This indicator incidentally proved to be most attractive to these flatworms and they ingested large amounts of food stained by it; particles of the indicator dropped into culture dishes attracted the flatworms to them and stimulated protrusion of the pharynx.

(3) Experimental feeding with fat

Large amounts of cod liver oil suspensions stained with Sudan IV were readily ingested from carrier gammarids, often increasing the body volume of the flatworms by 40–50%. Saline squashes and frozen sections prepared thirty minutes after feeding showed some stained oil globules within the columnar cells, and the number increased with time. Irrigation of the preparations with Nile Blue (Smith, 1907; George, 1951) showed the globules changing from red to deep blue as the stain penetrated the cells showing that much of the phagocytosed oil was undergoing intracellular lipolysis with the production of free fatty acids. Nothing suggesting intraluminar lipolysis was seen. Stained globules appeared early in the mesenchymal cells, and later became much more numerous, but only a few ever showed any color reaction with the Nile Blue. Since the Sudan IV stains the fatty acid radical of fat molecules and remains with it through lipolysis and resynthesis, it appears probable that the red globules in the mesenchyme cells had mainly been broken down and re-formed in the gut cells into the flatworm's own particular type of fat and then passed into the mesenchymal cells for storage. Flatworms with this stored fat had a diffuse pink color which persisted for several weeks and faded only gradually as the fat was metabolized and the Sudan IV excreted. Saline squashes made six weeks after feeding still showed occasional red globules in the mesenchymal and columnar gut cells. It was not possible to determine the pH conditions attending intracellular lipolysis since indicators dissolved in the water of the cod liver oil suspension were not taken up by the cells. Suet particles were also fed. The largest particles remained in the gut unchanged in size and shape until ejected 24 hours later, confirming the absence of intraluminar lipolysis. The smaller particles were phagocytosed and digested intracellularly.

The nature and location of the food reserves

Polycelis forms large reserves of protein and fat, supplemented by some carbohydrate reserve, and these enable the flatworm to survive for at least three months without food.

The gonads, mesenchyme and general body tissues constitute a protein reserve which is drawn upon during starvation, with a consequent reduction in body size, but there are also specific protein reserves in the sphere cells of the gastrodermis. These contain 8–16 small homogeneous concentrations which normally stain deeply with modified Millon, eosin and iron haematoxylin, but their appearance and staining affinity varies with the nutritive state of the animal. In well-fed laboratory individuals, and in those collected out of doors in late summer and well nourished in preparation for winter, the spheres are all compact and densely staining. During starvation they become increasingly indistinct, stain poorly and finally disappear. At the same time a progressive reduction in the volume of both columnar and sphere cells results after about three months in a flattened gastrodermis devoid of any inclusions. The significance of the sphere cells as protein reserve can be demonstrated by comparing the proportion of sphere and columnar cells under a variety of nutritive conditions. These are summarized in Table I which is compiled from cell counts of every third section of a number of histological series.

The fat reserve is laid down as globules in the inner mesenchyme and columnar

gut cells. It decreases only slowly during starvation, and significant amounts still remain after three months without food.

The carbohydrate reserve occurs as small irregular granules of glycogen, scattered throughout the mesenchyme and columnar cells. It decreases rapidly on starvation and disappears from the mesenchyme after about a fortnight. Small amounts persist in the gut cells, however, for much longer than this, but these may possibly result from the conversion of other reserves. A purely carbohydrate diet results in an enormous increase in the amount of glycogen in the mesenchyme and the flatworms, provided they were mature, survived as well on this diet as upon purely protein or fat diets.

The epidermal and subepidermal glands and their relation to feeding

These secretions can be divided into mucoid, giving a positive reaction with P.A.S. and Alcian Blue (Steedman, 1950), and the non-mucoid or rhabdoid, giving a negative reaction.

The mucoid can be further differentiated into eosinophilic and basophilic. The latter is produced by scattered gland cells in the epidermis and outer mesenchyme,

TABLE I

The proportion of cell types in relation to nutrition

Nutritive condition	Ratio of "sphere" to columnar cells
High protein diet	930:3723 = 1:4
Normal diet	625:3957 = 1:6.3
Starvation: 14 days	435:2957 = 1:6.7
Starvation: 28 days	371:3545 = 1:9.5
Starvation: 2 months	176:2668 = 1:15.5
Starvation: 3 months	"Sphere cells" absent and gastrodermis syncytial

and is used in gliding and to a minor extent in trapping prey as already mentioned. There are no large aggregations of basophilic glands as described in some other triclads. The much more adhesive eosinophilic mucus is produced both from scattered gland cells and from concentrations of mesenchymal gland cells, the "marginal adhesive glands" of Hyman (1951), situated along the borders of the ventral surface. During its discharge the epidermis becomes elevated into small temporary papillae so that the secretion is laid down in lines of oval deposits, from which are formed the main elements of the "snares."

The non-mucoid rhabdoids are transparent greenish rods, 15–20 μ in length, produced by mesenchyme cells and passed out to the epidermis. Once outside the body the rhabdoids take up many times their own volume of water ("hydrate") and fuse to form a semi-fluid gelatinous layer which closely invests the body. This material can be collected for examination in two ways. It can be removed from the living flatworm by drawing a fine glass needle a number of times across the dorsal surface of the body. Alternatively, immersion of the worm in strong sodium chloride solution causes the discharge of enormous numbers of rhabdoids and at the same time inhibits their hydrolysis, so that they can be fixed in 95% alcohol for histological examination. Irrigation of unfixed rhabdoids with water causes them to hydrate rapidly (Figs. 15 and 16), producing the same sort of gelatinous sheet as invests

the normal flatworm. The lowest critical concentration of salt to give discharge without hydration is about 3.75%; possibly hydration within the epidermal cells may be inhibited by some comparable combined effect of the cytoplasmic salts. The rhabdoid material is basic (pH 8.0–8.2), soluble in acids and alkalies, and stains strongly with Millon; apparently it is of a purely protein nature, as all fat and carbohydrate tests were negative. The hydrated material is not toxic to other animals, annelids and crustaceans surviving for long periods in contact with it, and it plays no part in feeding. It is distasteful to fish, pieces of earthworm smeared in it being rejected by sticklebacks, but its main function is probably mechanical, acting as a "fluid cuticle" to protect the epidermis from abrasion and bacterial and fungal attack whilst still permitting ciliary activity, and in life it is probably in a continual state of loss and renewal. There is also a rapid discharge of rhabdoids if a flatworm is injured, to give a protective clot-like effect over the region of the wound.

ACOELA

Convoluta paradoxa (2–3 mm. long and pear-shaped with a pronounced ventral curling of the broader anterior end) feeds upon small marine crustaceans, protozoa, diatoms and similar organisms which are captured by various methods, depending upon their size. Minute prey such as protozoa are captured by the flatworm gliding slowly over algal fronds or stones with the solid syncytial gut partially extruded through the mouth like a large pseudopodium and the food engulfed amoeboid fashion and passed back into the syncytium for digestion. Alternatively the flatworm may spend long resting periods attached to the substratum by the adhesive papillae of the tail and with the rest of the body slightly raised in a "sitting-up" position. If larger prey, such as crustaceans or their larvae, come sufficiently close, the flatworm rapidly extends its body and grasps the prey with the curved anterior margin, which is covered with abundant sticky mucus produced by the frontal and other subepidermal glands. The flatworm then curls up ventrally, starting from the anterior end, so that the prey is drawn beneath it and pressed into the mid-ventral mouth. This is very distensible and the food is ingested whole and intact. More rarely *Convoluta* captures actively swimming creatures whilst it is itself either swimming freely or gliding over the algal fronds, but in all cases capture and ingestion are extremely rapid and occupy only 5–10 seconds. The flatworm shows marked avoiding reactions when dead food is encountered; it feeds only upon living organisms and does not act as a scavenger.

The simple pharynx is a very short ciliated tube leading directly to the solid "gut"—a central compact or vacuolated syncytium, one-third to one-half of which can be protruded through the mouth (Fig. 5). Its cytoplasm is finely granular and contains many vesicular nuclei. All prey when ingested is enclosed in temporary vacuoles which follow no definite path, but merely move back inside the animal to come to rest in any part of the syncytium. Occasionally vacuoles containing digesting prey may be found in the mesenchyme, but the cytoplasm about these vacuoles is always distinct from the surrounding tissue and is apparently a temporarily isolated part of the digestive syncytium. As digestion progresses the food becomes disorganized and breaks up and in Crustacea only fragments of the exoskeleton remain when digestion and absorption are complete, some 18–24 hours after feeding (Fig. 6). These indigestible residues then pass to the mouth to be thrown out.

It was not possible to determine the pH conditions controlling digestion as *Convoluta* refused crustaceans stained with indicator. Attempts to recover food in various stages of digestion for pH examination also failed, as it inevitably became contaminated with mesenchyme, mucus and sea water.

Fat forms the principal food reserve in *Convoluta*, occurring in large amounts as globules of 2–3 μ diameter in the outer, more compact mesenchyme and in the digestive syncytium. Small amounts of glycogen also occur as irregular granules scattered throughout the mesenchyme and digestive tissue. There are no specific protein reserves.

RHABDOCOELA

(1) *Macrostomum* sp. (2–3 mm. long and 0.5 mm. broad) feeds upon minute fresh water crustaceans, annelids, nematodes, rotifers and large ciliate protozoans. The mouth and pharynx are capable of great distension and any small creature moving near the flatworm is seized and ingested whole. Only living food is taken and mucus plays no part in its capture.

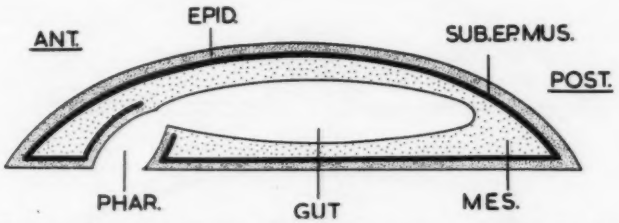


FIGURE 2. Diagrammatic longitudinal section of *Macrostomum* to show the simple pharynx. Abbreviations as in Figure 1.

The ventral slit-like mouth opens into a simple ciliated pharynx longer than that of *Convoluta* but still with no thickening of the underlying muscular layers (Fig. 2). Eosinophilic glands in the mesenchyme open into the pharynx but they are not mucus-producing and their precise function remains unknown. The gut is a simple unbranched sac extending almost the whole length of the body and its wall is made up of two kinds of cells, standing upon a thin basement membrane (Fig. 7). The larger and more numerous are columnar, some 30–40 μ tall and 5–6 μ broad, with basal vesicular nuclei and finely granular cytoplasm which may have inclusions, giving the cell a phagocytic appearance. The second type of cell is smaller and bears a close resemblance to the "sphere-cell" of the triclad gastrodermis. It is club-shaped, 15–20 μ tall and 3–4 μ broad distally, with the nucleus in the narrower basal region where the cytoplasm is often strongly basophilic. These cells contain 10–15 eosinophilic spheres which also stain with iron haematoxylin and modified Millon.

Sections of *Macrostomum* fixed 15 minutes after feeding on *Daphnia* showed the intact crustaceans lying free in the gut. In others fixed 10 hours later digestion was well advanced, with the muscles and organs greatly disorganized, whilst 24 hours after feeding only the exoskeleton remained, but as the latter retained its general shape there can be little movement or contraction of the gut during digestion.

This was confirmed by observation of living flatworms. During digestion the columnar gut cells became shortened, swollen and indistinct from one another, probably as a result of secretory activity or absorption of digested food, or both. The "sphere cells" remained unchanged. These observations confirmed the presence of intraluminar digestion in *Macrostomum* but after feeding upon *Paramecium* or small annelids almost all the gut cells showed discrete inclusions which were clearly particles phagocytosed from the disintegrating food mass in the gut lumen (Fig. 7). Thus digestion in *Macrostomum* must be a combination of intraluminar and intracellular processes, the latter occurring when the food in the lumen is sufficiently disintegrated for the particles to be phagocytosed. The small amount of phagocytosis and intracellular digestion seen with crustaceans may be due to the exoskeleton retaining most of the disintegrating material until it is rendered completely soluble. The pH conditions of digestion could not be determined as the flatworms refused to ingest prey stained with indicator.

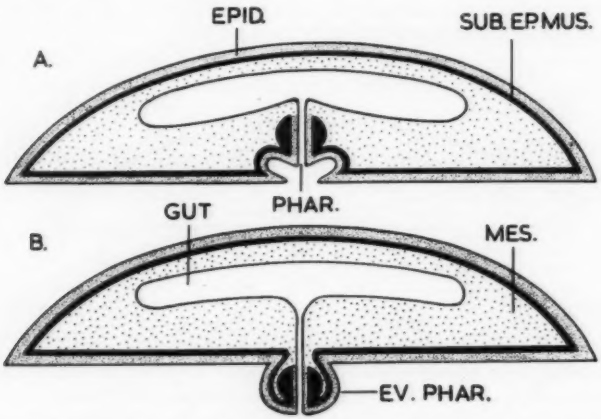


FIGURE 3. Diagrammatic longitudinal sections of *Mesostoma* to show the bulbous pharynx. A, The normal condition with the pharynx retracted. B, Pharynx everted for feeding. Abbreviations as in Figure 1.

Small amounts of glycogen and fat occur in the columnar gut cells and in the mesenchyme, whilst the "sphere-cells" of the gut epithelium appear to constitute a definite protein reserve, since they disappear after 10–14 days' starvation.

(2) *Stenostomum* sp. (1.5–3 mm. long and very slender) feeds mainly upon large ciliates and rotifers, which are seized in the anterior ventral mouth and engulfed whole. These animals are usually found by chance but the flatworm may search actively for them, crawling slowly forwards with the anterior end slightly raised and the mouth wide open. The latter is capable of great distension and is fringed with large cilia which probably help to sweep the prey back into the pharynx, where it is retained a few seconds for examination. If unsuitable it is rejected, but if acceptable the mouth is closed and the pharynx contracts violently to force the food into the gut. The pharynx is a simple ciliated tube, similar to that of *Macrostomum*, with some basophilic gland cells producing mucus to help ingestion. The

gut is a simple blind sac extending almost to the posterior end of the body, its anterior end is contracted and open communication with the pharynx exists only during actual ingestion. The gastrodermis is of unciliated club-shaped cells, 20–25 μ tall and 6–8 μ broad distally with basal nuclei and granular inclusions.

When ingestion is complete, and the pharynx again closed off, the gut contracts violently and the food is driven to and fro in the lumen (Fig. 9). Ciliates are broken up within fifteen minutes, and indeed are often disintegrated by the pharynx during ingestion. Rotifers resist longer but eventually their body contents are squeezed out through the mouth or some weak spot in the integument and the empty cuticle ejected. The resultant particles are phagocytosed by the gut cells and show for a time as eosinophilic inclusions until intracellular digestion is completed. Occasionally *Paramecium* stained with indicators covering a pH range of 3.5–8.4 were accepted but during their disintegration the pH remained unaltered, showing the absence of intraluminar digestive activity.

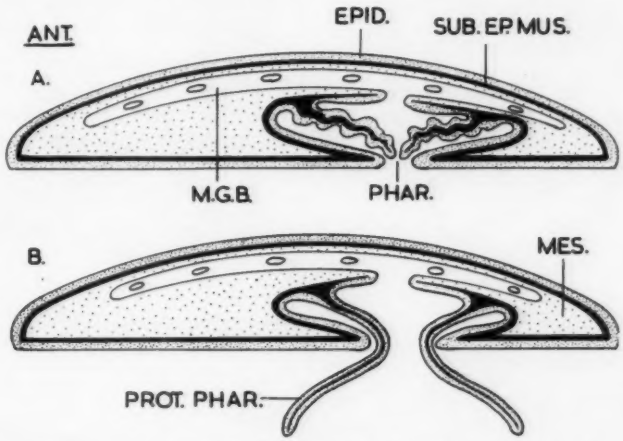
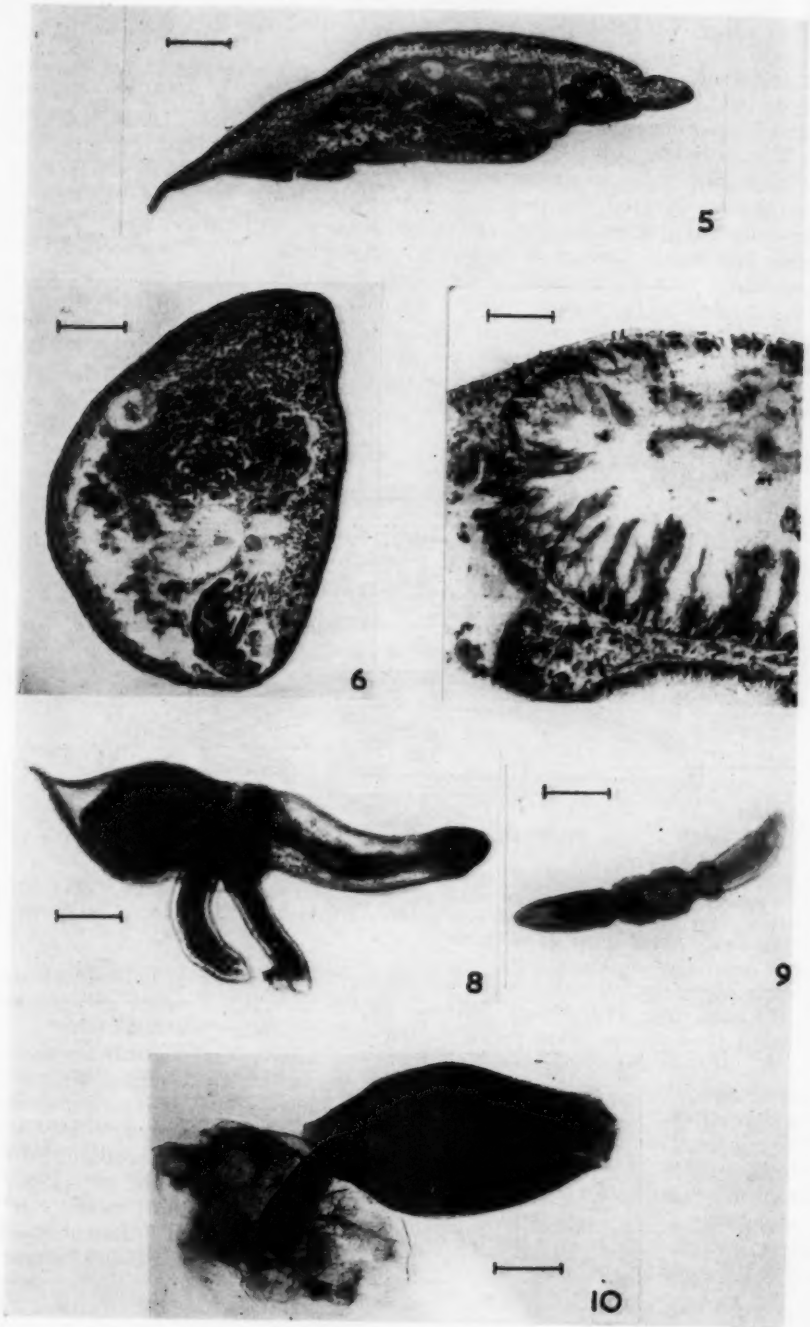


FIGURE 4. Diagrammatic longitudinal section of *Leptoplana* to show the ruffled plicate pharynx. A, The normal condition with pharynx retracted. B, The pharynx protruded to envelop food. Abbreviations as in Figure 1.

Fat forms the only food reserve in *Stenostomum*, occurring as small globules in the gut cells. The amount decreases rapidly and little remains after a week, which is about the maximum period the flatworm can endure without food.

(3) *Mesostoma tetragonum* (5–8 mm. long and 1–2 mm. broad) feeds upon small oligochaetes, crustaceans and insect larvae, which are captured directly by the flatworm wrapping itself around them. The bulbous pharynx is then protruded through the median ventral mouth (Fig. 3) and applied to the prey's body wall. Strong sucking movements made by alternate contractions and expansions of the radial musculature of the pharynx draw small prey bodily into the gut (Fig. 8). Larger animals are held until the body wall ruptures and the pharynx can be thrust into the body to suck out the contents, but in a somewhat inefficient manner so that frequently only the body fluids are withdrawn. *Mesostoma* is also a



FIGURES 5–10.

scavenger and will feed upon any sort of dead prey, if not excessively decomposed. The gut is a simple sac and the gastrodermis is syncytial, 15–20 μ thick, often with spherical inclusions.

The ingestion of whole prey implied that digestion in *Mesostoma* is at least partially intraluminar and this was confirmed by experimental feeding with clotted frog blood. The erythrocytes are largely broken up within thirty minutes, though their freed nuclei are still distinct, but there is no evidence of phagocytosis by the gut cells during this initial period. After three hours the material in the gut lumen has become a homogeneous mass, though a light reaction with Feulgen indicates that the ruptured nuclei are not yet entirely decomposed, and as this stage is reached material of similar appearance begins to show as small spheres within the gut cells. After six hours the lumen was empty and the gut cells packed with spheres, which in turn decreased and disappeared within 24 hours. Intracellular digestion in *Mesostoma* is therefore preceded by some degree of intraluminar digestion, but details are unknown as no successful method of feeding with indicators could be found.

POLYCLADIDA

(1) *Cycloporus papillosus* (1–2 cm. long and broadly oval) is usually found attached by its single ventral sucker to the surface of the encrusting colonial tunicates *Botryllus* and *Botrylloides*, and it appears to feed exclusively upon these animals. The anterior cylindrical plicate pharynx, shorter but otherwise resembling that of *Polycelis*, is protruded forwards and thrust down into the colony to suck out individual zooids. The pharynx leads into the median gut branch which gives off 6–8 pairs of lateral diverticula, subdividing in turn to give the typical polyclad arrangement. The distal gut branches lead up to large "anal chambers," ranged along the margins and opening by pores through the epidermis. In histological preparations the branches and anal chambers always appear closed off from each other, and no actual passage outwards of gut material has ever been found; possibly temporary openings may develop for the passage of excess water taken in with the food.

The majority of the gastrodermal cells are columnar, 35–40 μ tall and 5–8 μ wide, with their free margins ciliated, the cilia being particularly strong in the median diverticulum (Fig. 17). There is, however, an anomalous region of columnar cells at the posterior end of this diverticulum where the cilia are replaced by what appears to be a "brush border." Scattered amongst the columnar cells are smaller

FIGURE 5. Longitudinal section of *Convoluta* showing the central vacuolated syncytium. Haematoxylin and eosin. Scale 0.25 mm.

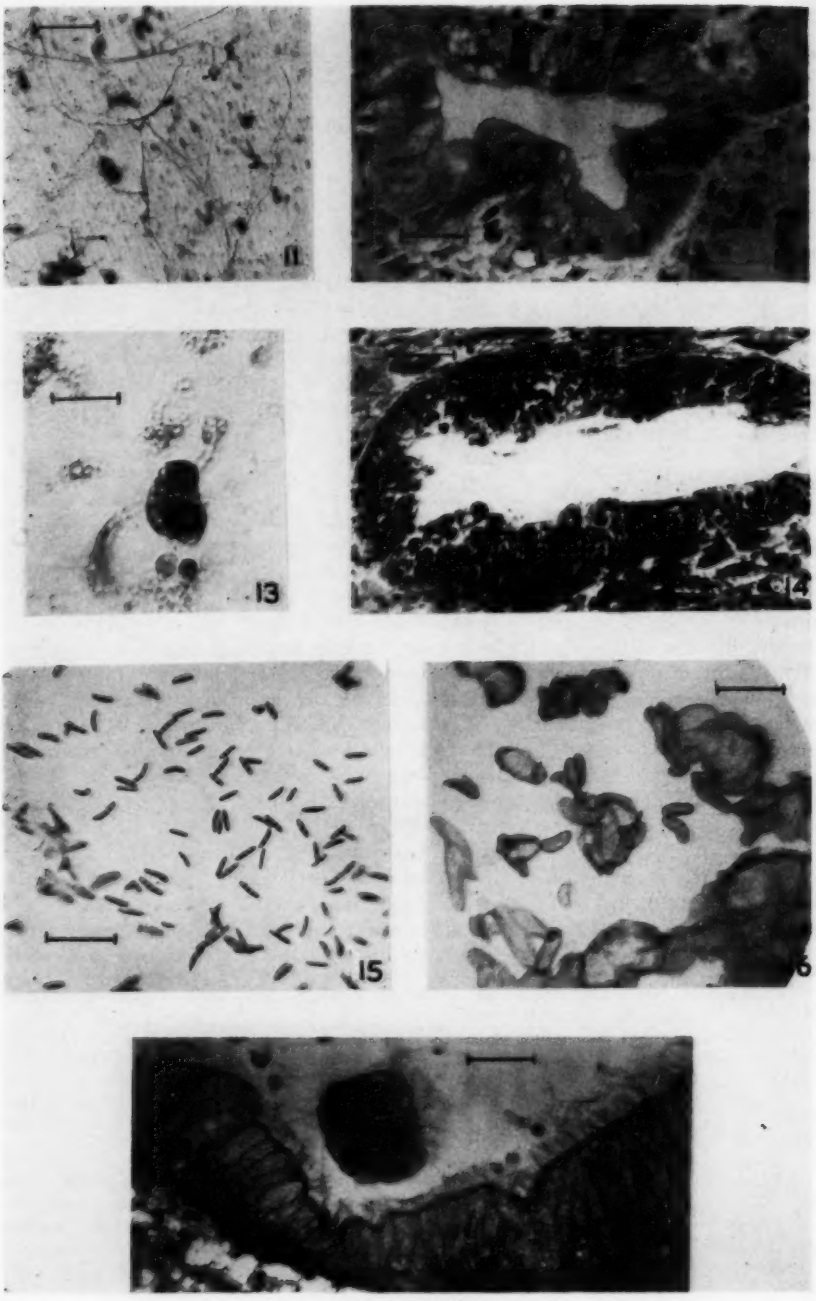
FIGURE 6. Transverse section of *Convoluta* showing a recently ingested intact crustacean and one completely digested except for the exoskeleton. Haematoxylin and eosin. Scale 0.1 mm.

FIGURE 7. Longitudinal section of *Macrostomum* showing part of the simple pharynx (left) and the gastrodermis with small, club-shaped "sphere cells" between the long, projecting phagocytic cells. Haematoxylin and eosin. Scale 20 μ.

FIGURE 8. *Mesostoma* ingesting an annelid. From life. Scale 1 mm.

FIGURE 9. *Stenostomum* immediately after ingesting a *Paramecium* stained with neutral red. The gut is beginning to contract to break up the ciliate. From life. Scale 0.4 mm.

FIGURE 10. *Polycelis* killed whilst feeding on a large *Daphnia*. Note the protruded pharynx. Steinmann's fixative and borax carmine. Scale 1 mm.



FIGURES 11–17.

numbers of spherical glandular cells, up to 45 μ in diameter, their cytoplasm laden with tiny granules. These cells occur in various sizes, the smaller appearing between the bases of the columnar cells and the larger nearer the lumen; occasionally they were seen free in the lumen, and their disintegrated remains were found both here and between the bases of the columnar cells. Their phases of growth and disintegration, however, could not be related in any way to the condition or amount of food in the gut and it is not at all certain that they are concerned in digestion. The columnar cells themselves, on the other hand, do appear to secrete juices into the lumen, becoming swollen and vacuolated soon after feeding.

In feeding, the tunicate zooids reach the gut almost undamaged; histological examination at intervals after an observed feed shows a progressive homogenization of the food, leading to complete absorption after about twelve hours. At no time is there any indication of other than intraluminar digestion. Confirmation came from feeding starved *Cycloporus* on *Botryllus* colonies injected with starch paste; Lugol sections showed digestion confined within the lumen, with unchanged starch never appearing within the gut cells. Food containing indicators, however, was consistently refused. Fat globules in the ciliated cells and mesenchyme constitute the only significant food reserve. Only minute amounts of glycogen occur in the mesenchyme and there are no specific protein reserves. The flatworm cannot withstand more than about seven days' starvation.

Only basophilic mucus glands are present, scattered throughout the epidermis and ventral mesenchyme, and mucus plays no part in feeding. Rhabdoid-producing cells are common in the mesenchyme, particularly between the posterior gut diverticula, and their cycle of activity can be followed in its entirety. Immature cells contain small eosinophilic granules and rods which enlarge into the fully formed rhabdoids. As these are discharged the cells shrink to a fraction of their former size and migrate into the nearest gut diverticulum; there they disintegrate and are presumably expelled with the unwanted food material. The discharged rhabdoids themselves follow one of two courses. Some pass to the epidermal cells and so to the exterior in the usual way. The experimental removal and examination of these rhabdoids gave results identical to those reported for *Polycelis*. Others aggregate to form conspicuous tracts, greyish white in the living flatworm, which converge upon the gonopore. The rhabdoids pass through or between the cells of the gonopore wall and are discharged with the eggs. Sections of the egg masses show the individual eggs to be embedded in a gelatinous matrix which has properties similar to those of hydrated rhabdoids. The rhabdoids are not concerned in any

FIGURE 11. Mucus "snares" produced by *Polycelis*. Alcian blue. Scale 0.5 mm .

FIGURE 12. *Polycelis*. Transverse section of gut diverticulum showing columnar phagocytic cells and smaller heavily staining "sphere cells." Iron haematoxylin. Scale 40 μ.

FIGURE 13. *Polycelis*. An isolated columnar cell with phagocytosed starch. From a saline squash. Lugol. Scale 40 μ.

FIGURE 14. *Polycelis*. Transverse section of the gut diverticulum 24 hours after feeding on chick blood, showing phagocytosed erythrocytes undergoing intracellular digestion. Iron haematoxylin. Scale 40 μ.

FIGURE 15. *Polycelis*. Rhabdoids discharged after immersion in 5% aqueous sodium chloride. Scale 50 μ.

FIGURE 16. *Polycelis*. Rhabdoids discharged as in Figure 15, then irrigated with tap water. Scale 50 μ.

FIGURE 17. *Cycloporus*. Longitudinal section of the median gut branch showing ciliated gastrodermis. Haematoxylin and eosin. Scale 40 μ.

way in the formation of the egg membranes since these stain strongly with P.A.S. and are already fully formed in the oviduct well above the point of entry of the rhabdoids.

(2) *Leptoplana tremellaris* (2–2.5 cm. long and pear-shaped) feeds upon polychaetes, isopods and amphipods which are captured directly without any trapping devices. The prey is seized by the anterior part of the flatworm's body and wrapped round by the expanded body margins. The flatworm then curls up and passes the prey back along the ventral surface to the median mouth. The pharynx is of the ruffled plicate type (Fig. 4), consisting of a large thin convoluted oval curtain suspended from the roof of the peripharyngeal chamber, and is protruded through the mouth to envelop the prey. Small animals are ingested whole, either by simple peristalsis or by the pharynx being drawn back within the mouth, a process requiring five minutes. Larger organisms are enveloped as far as possible and the pharynx is retracted slightly so that the prey is partially ingested; the flatworm then presses itself down on the substratum and the pharynx makes strong sucking movements which are often supplemented by movements of the whole body. This process may continue for an hour or more, whilst the prey is disintegrated and ingested piece by piece or has its integument ruptured and the contents squeezed out. *Leptoplana* also feeds upon dead animal material provided it is not too decomposed.

The gut is of the usual polyclad form but the gastrodermis here consists of unciliated vacuolar columnar cells. In well-fed individuals these are indistinct in outline or even syncytial and the cytoplasm loaded with eosinophilic spheres which disappear on starvation. This appearance of phagocytic activity was confirmed by experimental feeding with frog blood and small pieces of *Littorina* muscle. The former were rapidly taken up by the gut cells and their intracellular digestion and disappearance easily followed. The small pieces of muscle entered the gut entire but subsequent changes in their histological appearance showed that they were partially broken down by enzymatic activity in the gut lumen before entering the gut cells, where their fate follows that of the usual eosinophilic inclusions. In this case, therefore, intraluminar digestion is incomplete and is succeeded by phagocytosis and intracellular digestion.

The possibility that enzymes from the gut could be regurgitated to assist disintegration in the protruded pharynx was investigated by using pieces of *Littorina* foot muscle too large to be ingested whole. These pieces were removed from the flatworm's pharynx after a series of time intervals and checked against control muscle for pH and enzymatic changes. Control muscle was slightly acid (pH 6.6–6.8), but after ten minutes decreased to 5.4 and after twenty to 4.5. For proteolytic activity the muscle was laid on gelatin-coated slides and half an hour allowed for any enzyme to diffuse out. The gelatin below and around a piece of muscle which had been in the pharynx for a quarter of an hour was dissolved away and the holes formed in the gelatin film showed clearly after staining the slide with eosin. Control muscle had no such effect. Corresponding acidity and enzyme production in the gut lumen can therefore be reasonably inferred.

Fat again forms the only significant food reserve, and what has been said of *Cycloporus* about mucus and rhabdoids applies also to *Leptoplana*.

Discussion

The general pattern of flatworm nutrition consists more of a series of intimate relationships between the nature of the food, the structure and function of the pharynx, and the course of digestion, than of a simple increase in complexity from primitive to more elaborate forms. Possibly the "amoeboid" method of feeding in the Acoelan *Convoluta* is primitive to the group (*cf.* Hyman, 1951), and no longer found in other forms. Certainly the alternative method in *Convoluta* of using the body as a whole to envelop the prey is still retained in one form or another by the majority of flatworms, with its effectiveness enhanced by elaboration of the pharynx. The limitation of the simple pharynx, found also in the rhabdocoels *Macrostomum* and *Stenostomum,* is that prey has to be engulfed whole and its size is thereby restricted. In contrast, the bulbous pharynx of the rhabdocoel *Mesostoma* can not only ingest small animals but can be forced into larger animals to suck out their contents, though little triturating effect is possible. The cylindrical plicate pharynx of the triclad *Polycelis* is used in a similar but much more effective manner, the whole contents of the prey being withdrawn and discharged into the gut in a finely divided condition. The comparable pharynx of the polyclad *Cycloporus,* however, is used to deliver largely undisrupted food into the gut, the difference being reflected in the subsequent course of digestion. The ruffled plicate pharynx of the polyclad *Leptoplana,* though developmentally akin to the last, is used rather as an extension of the gut in which preliminary breakdown is enzymatic instead of mechanical. Thus the elaboration of the pharynx has made available to the flatworms an increasing range in the size and variety of their food. Mucus plays a minor part in the feeding of the majority, but does serve to hold the prey and facilitate ingestion. In *Polycelis* alone it is used for the actual capture of prey.

The course of digestion is linked with the feeding mechanism, since this determines the condition of the food on entry to the gut. In *Convoluta* the position is anomalous in that digestion in its gut syncytium might be looked upon either as intracellular or as occurring within temporary lumina. In *Polycelis* the food is very finely divided by the pharynx and the particles taken in by phagocytosis for intracellular digestion. No evidence of intraluminar digestion was found in this species, either of mixed food or of the three food elements fed separately, in agreement with Willier, Hyman and Rifenburgh (1925) and Kelley (1931); the contrary opinion of Arnold (1910) was based on the erroneous supposition that the "sphere cells" of the gut were glandular and discharged into the lumen. Some evidence of intracellular digestion in mesenchyme cells was observed, of food apparently received indirectly from the endoderm cells. In striking contrast is the purely intraluminar digestion of *Cycloporus,* which shows that the flatworms are capable of evolving this more advanced process. In all the other types examined some preliminary breakdown of the food, either by mechanical or enzymatic means, occurred in the pharynx or in the gut lumen, to be followed by phagocytosis and intracellular digestion; this preliminary breakdown should perhaps be interpreted as an adaptation to allow the persistence of the more primitive form of digestion.

Experimental feeding with proteins, carbohydrates and fats given separately showed that fully grown *Polycelis* can not only utilize and store all these food elements but can survive upon any one indefinitely. *Polycelis* also forms normal food

reserves of all three types: specific protein reserves in the "sphere cells" of the gut, fat and glycogen in the gut and mesenchyme cells. Glycogen is the first to be used up and appears the least important. In the others fat forms the main food reserve, supplemented by protein in *Macrostomum,* but they are less prominent than in the triclad, and the capacity to withstand starvation is correspondingly reduced.

I wish to thank Professor E. A. Spaul for suggesting this problem and for assistance, and Dr. T. Kerr for constant guidance and encouragement.

Summary

1. A study has been made of the food, feeding mechanisms, digestion and food storage in the triclad *Polycelis cornuta,* supplemented by observations on representatives of the other three flatworm orders.

2. Each form has a characteristic method of feeding, but in general the range of available prey has been greatly increased by elaborations in the structure and use of the pharynx. Mucus plays a minor part in feeding, except for the "snares" of the triclad. Rhabdoid material has no function in feeding. It forms a temporary cuticle and in polyclads a covering for the eggs.

3. In *Polycelis* mechanical breakdown of the food is followed by phagocytosis into the gut cells and intracellular digestion. Elsewhere the preliminary breakdown may be wholly or in part enzymatic; only in *Cycloporus* is there purely intraluminar digestion with absorption in place of phagocytosis.

4. *Polycelis* can make use of all three food elements, and stores of each type normally occur. In the other forms food storage is less well developed though reserve fat at least is usually to be found.

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VARIATION OF NITROGEN AND CARBOHYDRATE CONSTITUENTS DURING THE DEVELOPMENT OF HIMANTHALIA ELONGATA (L.) S. F. GRAY

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Seasonal variations in the chemical composition of brown algae have been studied by various workers (Lapicque, 1919; Lunde, 1940; Black, 1948a, 1948b, 1949, 1950a, 1950b, 1954a, 1954b; Channing and Young, 1952, 1953; Øy, 1951; Smith and Gordon Young, 1953; Wort, 1955). Black and Dewar (1949) correlated the physical and chemical properties of the sea water with the chemical constitution of algae. In all these investigations, however, no indication of the age, stage of development or sex of the plants is given.

Moss (1950) found that marked changes in the chemical composition of *Fucus vesiculosus* were associated with the development of the reproductive structures. In later work Moss (1952) noted that during the Spring a variation in chemical constituents occurred in several developmental stages of *Himanthalia elongata* collected at the same time from the same habitat.

Black (1954a) has shown that at certain times of the year gradients of carbohydrates, proteins and mineral matter occurred along the frond of *Laminaria saccharina*. Studying the variation of nitrogen in *F. vesiculosus* and *L. saccharina*, Jacobi (1954) observed horizontal and vertical gradients of nitrogen, water content and fresh weight. During the time of sporulation the frond of *L. saccharina* showed a higher percentage of soluble nitrogen than during the time of vegetative growth. Jacobi suggested that ontogenetic factors were more decisive in bringing about variations in chemical composition of brown algae than the availability of nutrients in the sea water as postulated by Black and Dewar (1949). For *Himanthalia elongata* similar variations in chemical constituents occur along the length of the plant (Jones, 1956). The same author noted that the mature vegetative tissues of the plant were lower in nitrogen content than the actively growing reproductive tissues, and that the male receptacles were higher in nitrogen content than the female receptacles.

No work appears to have been carried out to determine the variation of chemical constituents during the growth and development of brown algae from germination to maturity and senescence. For such a study, therefore, the brown alga *Himanthalia elongata* has been selected, for it enables an independent study to be made of the variation in chemical composition of both vegetative and reproductive tissues during the growth and development of the plant.

Materials and Methods

Collection of material

Samples of *Himanthalia elongata* were collected from St. Mary's Island, Northumberland, at low water as the tide receded. All samples were returned to the

laboratory in large glass vessels, thus ensuring that the plants, prior to analysis, were fully turgid. During 1954, the following major stages of growth and development of the plant were investigated:

1) The development of the young vegetative buttons until they produced receptacles at the end of the year. At St. Mary's Island, the young receptacles made their appearance during September to October.

2) The maturation of the plants in their second year of growth. During this period the receptacles grow rapidly in the summer months and develop conceptacles containing the gametes.

For analysis, samples were sorted into their respective stages of development. Each sample contained at least 100 individuals. Wherever possible the vegetative buttons were separated from the receptacles and analyzed separately so that variations in chemical composition throughout the development of both vegetative and reproductive tissues could be studied. Because of the difference in nitrogen content of male and female receptacles of *Himanthalia* (Jones, 1956), for the mature plants only female ones were used.

Analytical procedure

Methods of analysis were those described previously (Jones, 1956). Fucoidin as combined L-fucose, however, was estimated by the improved method of Black, Cornhill, Dewar, Percival and Ross (1950).

Expression of experimental results

For the purpose of this study it was considered that the results would lend themselves best for interpretation if they were expressed on a unit plant basis.

Results

1. *Young developing plants*

During the development of the young vegetative button there is a gradual increase in fresh and dry weight until the young receptacles make their appearance in October (Table I). Thereafter the weight of the button remains comparatively steady. The receptacles, however, increase in both fresh and dry weight after their initial appearance.

The total nitrogen of the young plants gradually increases throughout the year. In the button the protein content steadily increases, while the non-protein nitrogen increases with the growth of the button until October, when, at the time of receptacle initiation, it falls appreciably (Fig. 1). The relationship between the protein and non-protein is illustrated in Figure 2. During the period January to August the ratio rapidly decreases, suggesting a building-up of non-protein constituents. From August to the end of the year, the ratio increases indicating a synthesis of protein. This synthesis precedes the development of the young receptacles, which are themselves rich in protein and non-protein nitrogen. The build-up of a soluble non-protein reserve, of which peptide nitrogen is the chief constituent, is therefore clearly indicated during the early stages of the development of the button.

TABLE I

Variation of constituents during the development of young plants, 1954

Month		Weight of plants gm./unit plant		Nitrogenous constituents mg./unit plant						Carbohydrate constituents mg./unit plant				Total ash mg./unit plant
		Fresh	Dry	Total N	Protein N	Non-protein N	Volatile base N	Free amino N	Peptide N	Alginic acid	Mannitol	Laminarin	Fucoidin	
January	B	0.54	0.07	1.02	0.87	0.15	0.04	0.04	0.07	13.9	3.9	1.4	5.1	28.0
February	B	0.72	0.11	1.48	1.25	0.23	0.06	0.06	0.10	—	12.1	—	—	—
March	B	—	—	—	—	—	—	—	—	—	—	—	—	—
April	B	0.78	0.14	2.02	1.68	0.34	0.08	0.09	0.15	26.1	13.6	2.7	10.0	56.0
May	B	—	—	—	—	—	—	—	—	—	—	—	—	—
June	B	1.69	0.24	3.71	2.79	0.92	0.31	0.15	0.35	43.8	32.4	5.1	18.6	91.0
July	B	2.00	0.32	4.62	3.58	1.04	0.45	0.24	0.61	56.0	41.5	7.0	20.5	112.0
August	B	1.80	0.31	4.31	3.06	1.25	0.38	0.15	0.50	58.3	43.1	12.3	28.0	108.0
September	B	1.96	0.38	4.67	3.67	1.00	0.36	0.19	0.50	64.8	47.8	34.5	31.4	110.0
October	B	1.62	0.33	4.44	3.69	0.75	0.21	0.25	0.31	71.5	36.6	27.0	20.7	97.0
November	B	1.76	0.36	5.16	4.24	0.92	0.17	0.14	0.33	82.0	25.1	21.5	22.5	120.0
November	R	0.15	0.03	0.56	0.33	0.23	0.02	0.02	0.03	4.0	2.0	—	1.7	8.0
December	B	2.00	0.36	5.32	4.58	0.74	—	—	—	83.0	24.4	11.1	26.0	127.0
December	R	0.32	0.04	0.91	0.53	0.28	—	—	—	6.6	2.4	0.5	2.6	13.0

B = Button.
R = Receptacles.

Throughout the growth of the young plant the alginic acid content increases even after the production of the receptacles. The other carbohydrate substances, namely mannitol, laminarin and fucoidin gradually increase during the early months of the year, then become more concentrated during the summer months, no doubt the result of active photosynthesis. Between August and October the vegetative but-

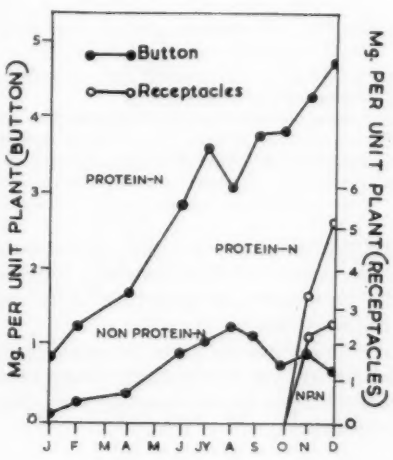

FIG.1

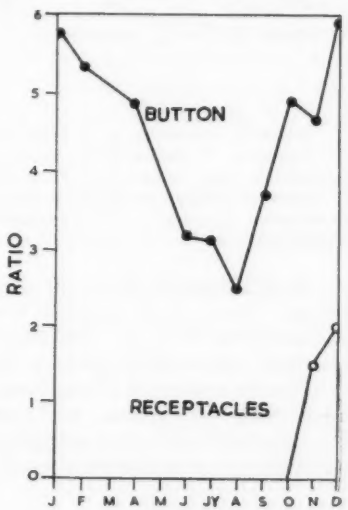

FIG.2

FIGURE 1. Variation in nitrogen during development of young plants.
FIGURE 2. Ratio protein: non-protein nitrogen during development of young plants.

tons exhibit a fluctuation in carbohydrates associated with the production of the receptacles. It is seen that the laminarin falls to a much lower level after receptacle formation in October. A similar fall in mannitol and fucoidin also occurs at this time. In the young receptacles the mannitol and fucoidin increase during the last two months of the year.

These latter results indicate the building-up of a reserve of carbohydrates throughout the early months of the year, which, with the increased photosynthesis during the summer, reaches a high concentration in the button. However, with the advance of the winter months this reserve is utilized for the rapid development of

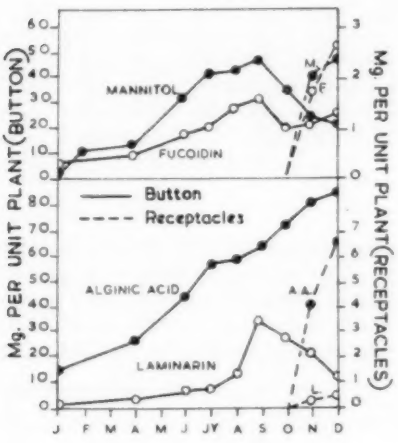

FIG. 3.

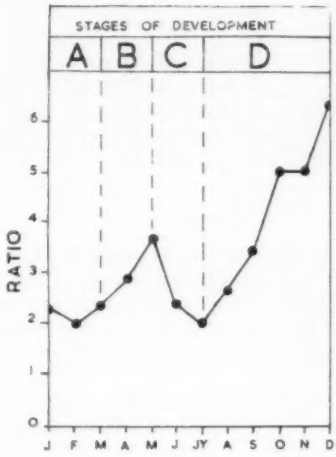

FIG. 4.

FIGURE 3. **Variation in carbohydrates during development of young plants.**

FIGURE 4. Variation in the ratio protein : non-protein nitrogen during development of receptacles in their second year of growth. A: Period of slow growth; conceptacles absent. B: Period of steady growth and development of conceptacles. C: Period of rapid growth and development of gametes. D: Cessation of growth, extrusion of gametes, and breakdown of receptacles.

the receptacle initials which, by repeated apical growth, give rise to the reproductive thongs. Concomitant with this there is, in the button, a fall in mannitol, fucoidin and laminarin (Fig. 3). The receptacles, after their appearance, carry on their own metabolic activity and synthesize their own carbohydrates.

The ash content of *Himanthalia*, like that of most marine algae, is considerably higher than that found for land plants. With the growth of the young plants there is a relatively high rate of accumulation of minerals from the surrounding sea water, particularly during the early months of development.

2. *The mature plants*

In the mature plants, during the second year of growth, the button remains relatively constant in fresh and dry weight (Table II). The receptacles, however, ex-

TABLE II

Variation of constituents during development of mature plants, 1954
(*The vegetative button*)

Month	Weight of plants gm./unit plant		Nitrogenous constituents mg./unit plant						Carbohydrate constituents mg./unit plant				Total ash mg./unit plant
	Fresh	Dry	Total N	Protein N	Non-protein N	Volatile base N	Free amino N	Peptide N	Alginic acid	Mannitol	Laminarin	Fucoidin	
January	2.19	0.39	4.95	4.07	0.88	0.21	0.21	0.53	83.3	39.0	18.0	42.4	125.0
February	2.51	0.45	5.68	4.77	0.71	0.15	0.17	0.42	—	37.8	—	42.6	162.0
March	2.17	0.40	5.40	4.47	0.83	0.16	0.16	0.38	88.4	22.1	—	23.5	147.0
April	1.88	0.38	4.58	4.00	0.58	0.12	0.12	0.33	88.7	25.6	8.1	—	136.0
May	2.17	0.40	5.02	4.29	0.73	0.13	0.22	0.40	88.7	28.5	—	23.3	144.0
June	1.97	0.37	4.92	4.29	0.63	0.13	0.20	0.31	83.4	32.8	—	27.9	138.0
July	1.95	0.40	5.60	5.06	0.54	0.12	0.21	0.32	88.0	30.6	—	—	116.0
August	1.95	0.38	5.41	4.85	0.56	0.10	0.19	0.25	84.0	29.1	9.5	33.9	105.0
September	—	—	—	—	—	—	—	—	—	—	—	—	—
October	1.81	0.35	4.74	4.23	0.51	0.11	0.26	0.20	77.5	21.5	—	—	100.0
November	1.62	0.33	4.81	4.37	0.44	0.13	0.18	0.18	75.2	20.9	7.3	30.5	95.0
December	1.64	0.33	4.77	4.32	0.45	—	—	—	—	—	—	—	—

hibit marked changes. During the early months of the year, growth of the receptacles, as determined by the fresh and dry weights, is slow, but steady. In the spring and early summer there is an increase in both fresh and dry weights, reaching a climax during the month of July, when the fresh weight of the receptacles has increased some five-fold. This increase is not due to water uptake and turgor extension of the cells alone because a similar five-fold increase in the dry weight also occurs (Table III). After the month of July, with the approach of the winter months, the fresh and dry weights of the receptacles begin to fall. From October to December this decrease is rapid. The receptacles undergo several anatomical changes during this period of growth. For the first two months of the year the receptacle tissue is non-fertile. From March to May, conceptacles develop. From May to July there is a rapid development of conceptacles, terminating in the pro-

TABLE III

Variation of constituents during development of mature plants, 1954
(*The receptacles*)

Month	Weight of plants gm./unit plant		Nitrogenous constituents mg./unit plant						Carbohydrate constituents mg./unit plant				Total ash mg./unit plant
	Fresh	Dry	Total N	Protein N	Non-protein N	Volatile base N	Free amino N	Peptide N	Alginic acid	Mannitol	Laminarin	Fucoidin	
January	0.67	0.08	2.24	1.56	0.68	0.07	0.08	0.23	—	—	—	—	—
February	1.19	0.14	3.85	2.50	1.35	0.10	0.15	0.42	—	11.2	—	—	—
March	1.58	0.19	5.32	3.73	1.59	0.14	0.22	0.60	30.6	18.7	2.2	6.3	81.0
April	2.14	0.27	7.63	5.65	1.98	0.17	0.32	0.60	34.6	23.2	2.6	10.8	134.0
May	9.76	0.87	24.57	19.11	5.46	1.04	1.16	1.91	143.9	59.4	6.9	28.5	446.0
June	26.24	2.81	78.51	55.38	23.13	7.64	3.85	8.46	477.4	152.5	31.8	82.9	1324.0
July	112.80	14.97	347.00	231.14	115.86	40.00	21.26	41.62	2776.5	938.5	308.4	473.0	6719.0
August	101.50	13.37	251.49	182.50	68.99	14.97	11.17	23.40	2535.0	1458.8	266.0	764.8	6000.0
September	95.80	13.89	243.91	189.46	54.45	12.83	10.69	21.81	2868.3	830.1	314.3	923.5	6376.0
October	89.50	12.28	203.47	169.21	34.26	7.00	9.70	11.79	2773.5	746.6	391.7	—	5511.0
November	58.62	7.60	126.38	105.81	21.20	3.57	3.80	8.44	2009.4	266.0	195.3	—	3405.0
December	16.00	2.05	34.60	29.86	4.74	—	—	—	540.8	51.3	43.3	—	906.0

duction of antheridia and oogonia. By August the receptacles are "ripe" throughout their entire length, except for some 5 to 6 cm. above the button which remains sterile. Growth ceases and there is a gradual extrusion of the gametes in a basipetal sequence. Disintegration from the tips of the receptacles then follows. This occurs comparatively slowly over the months of August to October, but thereafter very quickly, so that by January of the next year only a few plants remain, the majority having completely disintegrated.

Very little change, if any, occurs in the nitrogenous constituents of the button in the second season after it has reached maturity. Most of the nitrogen in the button is in the form of protein; very little non-protein nitrogen is present. The receptacles show great fluctuations in their nitrogen content (Table III). The protein and non-protein nitrogen increases steadily during the early months of the year, but from March to July there is a rapid increase, particularly in protein. From August to the end of the year the nitrogen content decreases. The ratio protein : non-protein nitrogen (Fig. 4) shows a correlation between the ratio and the stage of development reached by the receptacles. During the early months of the year when growth of the receptacles is slow but steady, the ratio gradually rises in favor of protein synthesis within the receptacles. Between May and July the ratio falls sharply and this is coincident with the period of rapid growth and development of gametes within the conceptacles. This suggests that the early synthesis of protein is associated with extra conceptacle tissue, and that, at a later date, this protein is broken down to soluble constituents and transported to the conceptacles where re-synthesis takes place in the production of the gametes, resulting in the increase in the ratio from August to the end of the year. From October onwards the gradual extrusion of the gametes and the wearing away of receptacle tissue keep pace with one another and the ratio continues to rise. It is seen that the peptide nitrogen exceeds the free α-amino nitrogen in the soluble non-protein constituents of the mature receptacles.

During the production of the receptacles there was a proportion of the soluble non-protein nitrogen which could not be accounted for. Nitrate nitrogen could not be detected in the plants at any stage of development. It was therefore considered to be some nitrogenous substance associated with reproduction such as nucleic acid.

In the button the carbohydrates remain relatively constant throughout the year. Like the young vegetative button the "old" tissue remains high in alginic acid content. The mannitol is low, similarly laminarin and fucoidin, the latter being higher than the laminarin and similar in magnitude to the mannitol. Throughout their growth the receptacles are particularly low in fucoidin and laminarin. Alginic acid is the major constituent of the receptacle tissue, mannitol being slightly lower. During the early months of the year there is little change in the content of alginic acid and mannitol. However, with increased photosynthesis in the spring and summer months both these constituents increase rapidly. After the extrusion of the gametes the alginic acid and mannitol are considerably reduced.

With the increased growth the accumulation of inorganic ions by the developing receptacles occurs during the months of January to July and thereafter falls following the disintegration of the tissue. The mature button remains comparatively uniform in mineral ash content throughout the year, although there is a tendency toward a falling-off during the latter part of the plant's existence.

General Discussion

During the present investigation, variations in chemical constituents have been followed throughout the development of *H. elongata*. Other workers have generally selected their plants at certain times of the year, or have used well established and fully developed plants for their investigations, failing to include the young stages. This is particularly the case where the grapnel has been used for collection, for this method selects only the larger algae. The number of individuals analyzed from one habitat is also of great importance. The results obtained by Black (1949, 1950b) for the Laminariaceae were based on results of the chemical analysis of two plants per month. Baardseth and Haug (1952) have since shown that certain constituents of marine algae vary considerably from one plant to another in a uniform

Table IV

Expression of results obtained for the receptacles of mature plants during the months of April and August 1954

Month	Protein N	Ash	Alginic acid	Mannitol	Laminarin	Fucoidin
a. Results expressed as mg. per gm. dry weight						
April	21.04	500.0	128.0	86.6	13.4	39.9
August	13.65	445.7	189.6	109.6	19.9	32.2
Month	Protein N	Ash	Alginic acid	Mannitol	Laminarin	Fucoidin
b. Results expressed as mg. per unit plant						
April	5.63	134.0	34.6	23.2	2.6	10.8
August	182.50	5959.0	2535.0	1458.8	266.0	430.5

population, even when collected from the same habitat. The ash and alginic acid contents of 55 *L. digitata* plants which they collected at one time showed variations as great as those obtained by Black throughout the whole year.

The majority of studies concerning the chemical composition of marine algae have been carried out by chemists concerned with the possible utilization of algae for industrial purposes. These studies, based on the variation of chemical constituents expressed as a percentage of the dry weight, have lead to considerable misconceptions of the physiological problems of growth and development in marine algae. For example, Black (1948a, 1948b, 1949, 1950b) found that the Laminariales and the Fucales exhibit a decrease in "crude protein" content expressed as a percentage of the dry weight, during the period of rapid growth. He attributed this decrease of protein to the lack of nitrate in the sea water at that time of the year. One can hardly accept the statement made by Black (1950b, p. 52) that "rapid growth of the plant results in a decrease in the crude protein content." Where an increase in growth occurs, increase in protoplasm must be the determining factor. An increase in protoplasm with a decrease in protein is difficult to imagine, particularly as protein is one of the major components of protoplasm.

At the time of maximum growth in *Laminaria* and *Fucus* the carbohydrates and other substances, particularly ash, are high. An increase in these other substances will result in an *apparent* decrease in the protein content expressed on a dry weight basis. Had Black expressed his results on a unit plant basis a truer picture of growth and the variations accompanying growth and development would have been made, as the examples for the receptacles of *Himanthalia* illustrate (Table IV). A decrease in protein, ash and fucoidin content between April and August occurs when the results are expressed on a dry weight basis. In the actual plant, however, these constituents have greatly increased over this period (Table IVb). As a result of such an increased synthesis of metabolic products the plant has increased in size, form and reproductive development.

Parke (1948), in her studies on the growth of *L. saccharina*, found that the plant reached its maximum weight during June to July after the period of rapid growth. Plants were found reproducing at all months of the year, but the greatest number were found during October and March. Black (1948b, 1949) found that for the fucoids the maximum dry weight of the plants occurred during the period May to June. For *Himanthalia*, the present author recorded maximum fresh and dry weight values during the months July and August. At all these times of maximum weight the plants commence their reproductive phase, producing either spores or gametes. The development of the reproductive tissues will therefore exert a considerable influence upon the chemical composition of the plants.

Moss (1950, 1952) showed that for *F. vesiculosus*, and later for *Himanthalia elongata*, reproduction definitely influenced the chemical composition of the plants. Jacobi (1954) found that when *F. vesiculosus* and *L. saccharina* were grown under favorable conditions, seasonal variations in nitrogen similar to those published by Black occurred, and that prior to reproduction a mobilization of protein was evident. In *L. saccharina* a higher percentage of soluble nitrogen was recorded for the reproductive tissue than the adjacent vegetative tissue. This lead Jacobi to conclude that the increase in nitrogen content of the plant was most probably due to the development of the reproductive tissues which are higher in nitrogen during spore production or gamete formation.

The present work on the change in chemical composition during the growth and development of *Himanthalia* substantiates the findings of Moss (1952) and Jacobi (1954). During the first year of growth the young vegetative plants gradually build up a reserve of nitrogen and carbohydrate constituents which are utilized for receptacle production. The young receptacles, after their initiation in October, continued to grow steadily until the following year, when, during the months of April to July, they grew very rapidly and reached maturity. The fucoidin, mannitol and laminarin, accumulated during the summer months in the young vegetative buttons, on the appearance of the young receptacles, were greatly depreciated, suggesting they were utilized in the production of reproductive tissues. Associated with the receptacle formation there was a rapid synthesis of protein from the non-protein constituents. In the button and also in the developing receptacles the alginic acid content continued to increase. After the establishment of the receptacles the composition of the vegetative button throughout the second year of growth remained relatively constant.

With the increase in the sea temperature and the increased photosynthesis

during the early summer months active metabolism resulted in the extensive growth of the receptacles. The two polysaccharides, fucoidin and laminarin, were very low during this period. Alginic acid, mannitol and mineral ash, however, continued to increase. Utilizing the inorganic nitrogen sources of the surrounding sea water, the receptacles synthesized amino acids and peptides which were later utilized in protein synthesis during the formation of the gametes in the conceptacles. The plant by August reached maturity and the gametes were extruded in a basipetal sequence. This process was accompanied with one of disintegration of receptacle tissue, resulting in a reduction in the chemical composition of the receptacle, until, by December, little remained other than the sterile bases attached to the senescent button. The whole plant then quickly decomposed.

The high ash content of the tissues, particularly of the receptacles, is of interest for the ash content of land plants is usually only represented by about 5 per cent of the dry matter of the plant (Thomas, 1949). Unlike land plants, marine algae are immersed in their nutritive medium, the sea water. In many cases seaweeds concentrate elements several thousand times the concentration found in the surrounding sea water (Black and Mitchell, 1952). The mineral ash consists of inorganic salts, presumably in solution in the cell sap, and cations in combination with such organic substances as alginic acid and fucoidin. Again the reproductive tissues exhibit differences from the vegetative tissues of the plant, insofar as they were found to be considerably higher in ash content. The receptacles contained as much as 50 per cent of their dry weight in mineral ash. Similar high results were found for the receptacles of *F. vesiculosus* (Moss, 1950), particularly when the receptacles were ripe.

The effect of the onset of reproduction upon the chemical composition of plants is not only to be found in marine algae. Variations in nitrogen with development of reproductive tissue have been found to occur in land plants. McCalla (1933), growing wheat in water cultures, found a decrease in nitrogen in the vegetative parts of the plant after the ears had emerged. These findings were later confirmed by Miller (1939) who showed that from the end of May onwards the inflorescences of winter wheat were the only aerial parts of the plants to gain in nitrogen, whereas the stems and leaves lost their nitrogen progressively. Blanck, Giesecke and Heukeshoven (1933) and Blanck and Giesecke (1934) found that maturation of the oat plant was accompanied by a redistribution of nitrogen, which first decreased in the roots and later in the stems and leaves while, on the other hand, it accumulated in the inflorescences. In the barley plant Richards and Templeman (1936) also indicated a pronounced movement of nitrogen from the vegetative parts to the developing grain.

It appears, therefore, that in the life history of *Himanthalia elongata* from germination to maturity and senescence separate parts of the plant undergo their own ontogenetic changes which greatly affect the chemical composition of the plant.

The author wishes to thank Dr. Betty L. Moss, under whose supervision this work was carried out, for helpful advice in the preparation of the manuscript, and to express his appreciation of a research grant from the Institute of Seaweed Research which enabled the work to be undertaken. The author is also indebted to the Director, Dr. F. N. Woodward, for permission to publish.

Summary

1. Monthly variations in chemical constituents have been followed throughout the development of *Himanthalia elongata*.

2. Nitrogen, laminarin, mannitol, alginic acid and fucoidin increase during the first year's growth of the vegetative button.

3. Associated with receptacle initiation, the carbohydrate and nitrogen reserve of the button is utilized and there is a synthesis of protein from the non-protein constituents.

4. The young receptacles build up a reserve of non-protein materials which are later utilized in protein synthesis during the formation of the gametes.

5. It was concluded that during the life history of *Himanthalia elongata* from germination to maturity and senescence, separate parts of the plant undergo their own ontogenetic changes which are correlated with changes in the chemical composition.

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STUDIES ON SHELL FORMATION. VI. THE EFFECTS OF DINITROPHENOL ON MANTLE RESPIRATION AND SHELL DEPOSITION [1, 2]

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Shell formation in mollusks concerns the elaboration of an organic framework or matrix and the orderly growth of calcium carbonate crystals within this matrix. This matrix is composed of three fractions: a water-soluble protein, a scleroprotein, and a polypeptide (Grégoire, Duchâteau and Florkin, 1955). Synthesis and secretion of matrix and any active transport of inorganic ions involved in shell deposition will require metabolic energy. Glycolysis (Humphrey, 1950) and the utilization of tricarboxylic acid cycle substrates (Humphrey, 1947; Humphrey and Jeffrey, 1954; Cleland, 1951; Jodrey and Wilbur, 1955) have been demonstrated in oyster tissues and would lead to the formation of high energy phosphate compounds. If these high energy phosphate compounds are utilized in shell deposition, then dinitrophenol (DNP) by preventing phosphorylation (Hunter, 1951; Lardy and Wellman, 1953; Shacter, 1955) or reducing the tissue concentration of the compounds already formed (Hunter, 1951; Shacter, 1955) may be expected to retard shell deposition. This problem has been examined in the oyster *Crassostrea virginica*. The action of dinitrophenol was studied with respect to (1) respiration of shell-forming tissue; (2) calcium deposition using radioactive calcium as an indicator; and (3) shell regeneration in the whole oyster.

Methods

Measurements of the effect of DNP on respiration were carried out by the Warburg method using a strip of tissue about one cm. wide taken from the posterior portion of the mantle. Endogenous respiration was measured in sea water for 30–60 minutes. DNP was then added from the sidearm, and measurements were continued for periods of one to six hours. DNP solutions were buffered at pH 8 with 0.03 *M* glycine.

The isolated mantle-shell preparation (Hirata, 1953) was utilized for measurement of Ca^{45} deposition as described by Jodrey (1953). These mantle-shell preparations were placed in one liter of sea water containing DNP for one hour. Ca^{45} (one ml. of high specific activity) was then added and the preparations remained in this solution for an additional six hours. Reversibility of DNP action on calcium deposition was tested by first immersing mantle-shell preparations in DNP solu-

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[2] We wish to express our appreciation to Mr. Tadashi Tsujii for microscopic observations in the regeneration experiments.

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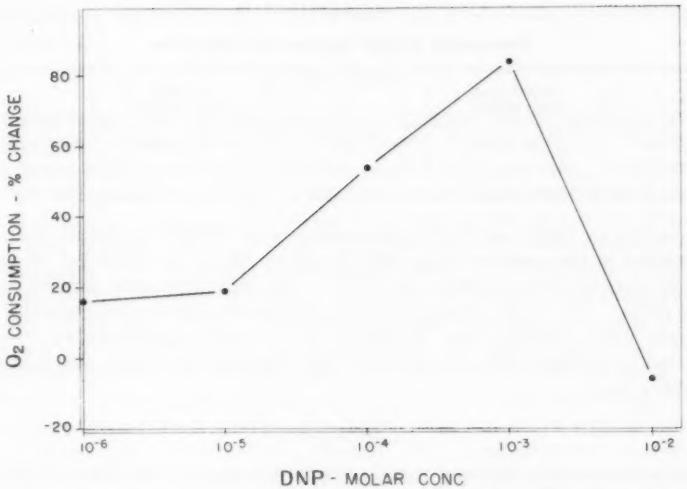

FIGURE 1. Effect of DNP on mantle respiration. Tissue weight, 180–220 mg. wet weight; temperature, 25.6° C.; pH 8.0; DNP, 0.2 ml. in sidearm; total volume 2.2 ml.; gas phase, air; salinity, 33.6–35.4 ‰; mean oxygen consumption of control, 15.6 μl O_2 per 100 mg. wet weight per hour.

tions for seven hours and then washing them in running sea water for two hours. The preparations were then placed in sea water containing Ca^{45} for six hours and the deposition of Ca^{45} was measured. Control preparations were carried through the same procedures but without DNP.

TABLE I

DNP effect on Ca^{45} deposition

DNP conc.	*n*	DNP added counts/min.*	*n*	No DNP counts/min.	P
10^{-3} *M*	5	35 ± 24	5	323 ± 179	<0.01
10^{-4} *M*	12	69 ± 37	12	193 ± 143	<0.01
10^{-5} *M*	9	376 ± 171	9	481 ± 205	0.3 > P > 0.2

Figures in columns three and five show mean calcium deposition by isolated mantle-shell preparations with standard deviations; *n* gives the number of cases. The concentration of Ca^{45} added per liter varied from 5.25 μc to 12.75 μc. For uniformity of presentation calcium deposition is expressed as counts per minute per 10 μc added per liter sea water per 6.2 cm.^{2} of shell. Temperature 25–26° C.; salinity 31.7–34.0 ‰. The experiments were not all carried out at the same time which probably accounts for the differences between the three mean values in column five.

* Shells without mantle were included along with the mantle-shell preparations in these experiments in order to obtain a measure of Ca^{45} exchange. However, it is not certain that the concentration of calcium for exchange is the same for both (Wilbur and Jodrey, 1952), and accordingly exchange corrections have not been made in the figures in Table I. When exchange values are subtracted from the deposition in the mantle-shell preparations the statistical significance is essentially that given.

TABLE II

Reversibility of DNP effect on Ca^{45} deposition

n	DNP added, then washed	n	No DNP, then washed	P
12	129 ± 93	12	87 ± 34	$0.2 > P > 0.1$

Details given in Methods and footnote of Table I. DNP concentration, 10^{-4} *M*.

The action of DNP on shell regeneration was studied on intact oysters with shells notched at the posterior edge and placed in 200 ml. or 1000 ml. of sea water containing various concentrations of DNP. All solutions were aerated and were changed daily. The extent of regeneration was determined microscopically.

Oysters were collected near Beaufort, N. C., and maintained for two weeks prior to use in natural waters or in tanks with running sea water supplied through hard rubber lines.

RESULTS

DNP caused a stimulation of mantle respiration at concentrations of 10^{-3} *M* and 10^{-4} *M* (Fig. 1). This increased respiration remained constant for at least six hours. The concentration of DNP which caused maximum respiratory stimulation (10^{-3} *M*, Fig. 1) gave essentially complete inhibition of Ca^{45} deposition by mantle-shell preparations (Table I). The same relationship of respiratory stimulation and inhibition of Ca^{45} deposition held true for 10^{-4} *M* DNP (Fig. 1 and Table I). In further experiments the inhibitory action of 10^{-4} *M* DNP on Ca^{45} deposition was found to be reversible (Table II). When the concentration of DNP was lowered to 10^{-5} *M* and 10^{-6} *M*, there was no statistically significant effect on mantle respiration (Fig. 1). Likewise, at 10^{-5} *M*, DNP had no significant effect on the deposition of Ca^{45} by the mantle-shell preparations (Table I).

Shell regeneration in the presence of DNP was inhibited as the concentration of the inhibitor was increased (Table III). The inhibitory concentrations of DNP were about the same for both calcium deposition by the mantle-shell preparation and for regeneration in the whole oyster (*cf.* Tables I and III). The last column in Table III indicates that DNP is toxic. However, of 20 oysters which were dead by the eleventh day of DNP treatment (all DNP concentrations), 10 showed regeneration. In those cases in which regeneration occurred, the structure of the

TABLE III

Effect of DNP on shell regeneration

DNP conc.	n	Oysters showing regeneration in DNP	Comments
0	10	10	No deaths in 14 days
10^{-5} *M*	10	8	1 dead after 14 days
5×10^{-5} *M*	10	7	9 dead after 11 days
10^{-4} *M*	10	3	9 dead after 5 days

Temperature 24.0–29.5° C.; pH 7.7–8.1; salinity 18.0–35.99 °/₀₀. Regeneration occurred in both valves in all but one case.

shell as observed microscopically was the same in DNP-treated oysters and oysters in sea water.

Discussion

The effects of DNP on the respiration of oyster mantle followed the general pattern as seen in many animal and plant tissues (Simon, 1953). Mantle respiration increased with increasing concentration of DNP, reaching a maximum 87% above the endogenous level. This degree of stimulation was considerably higher than that produced in the mantle by the addition of citric acid cycle intermediates (Jodrey and Wilbur, 1955). At concentrations of DNP which caused a respiratory stimulation, Ca^{45} deposition by the oyster mantle was reversibly inhibited.

The action of DNP may involve one or more of the following mechanisms concerned with shell deposition: (1) transport of calcium and carbonate ions across the mantle; (2) transfer of $CaCO_3$ crystals from the interior of mantle cells; (3) synthesis and secretion of the shell matrix; or (4) amoebocyte activities and shell regeneration. If the transport of the calcium and carbonate ions across the mantle requires the expenditure of energy, this active transport could well be inhibited by DNP (Taggart and Forster, 1950; Mudge, 1951; Levinsky and Sawyer, 1953). The transport of calcium carbonate crystals from the cell interior, as suggested by Bevelander (1953), might utilize an energy mechanism susceptible to the action of DNP. In the regeneration experiments failure of intact oysters to deposit matrix alone in the presence of DNP may be due to an effect on matrix synthesis since peptide bond and protein synthesis are known to be inhibited by DNP (Borsook, 1954; Tarver, 1954; Siekevitz, 1952). However, in view of the toxicity of DNP (Table III) one should not conclude that failure of regeneration reflects only a direct effect on matrix synthesis or secretion.

Another possibility arises from the fact that in the snail *Helix aspersa*, amoebocytes play a part in shell regeneration and are thought to be responsible for the deposition of matrix and calcium carbonate crystals (Wagge, 1955). However, the role of amoebocytes in oyster shell regeneration has not been studied and accordingly we do not know their significance with respect to the inhibition of regeneration by DNP.

Summary

1. Dinitrophenol stimulated oyster mantle respiration at 10^{-3} *M* and 10^{-4} *M*. Respiration was not significantly different from the endogenous rate at 10^{-2} *M*, 10^{-5} *M* and 10^{-6} *M* DNP.

2. Calcium deposition, as measured by Ca^{45}, was inhibited in isolated mantle-shell preparations at 10^{-3} *M* and 10^{-4} *M* DNP, the same DNP concentrations which stimulated respiration. Inhibition was found to be reversible.

3. Shell regeneration in whole oysters was inhibited by DNP. DNP concentrations which inhibited regeneration were toxic.

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TEMPERATURE DEPENDENCE OF BREATHING RATE IN CARP

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Temperature is an essential factor in the aquatic environment; it exerts a profound influence on the morphologic and physiologic characteristics of fishes. To infer from this the importance of temperature in adaptation, in its genetic meaning, seems to be logical.

An adequate study of this adaptation presupposes easily recognizable adaptive variants within a population. Our original intention was to work out an easy method to detect such physiologic variants which would not involve necessarily the death of the experimental objects, for breeding purposes. For several reasons—among which figure the data of Fox (1939) concerning the frequencies of respiratory movements with regard to the geographical distribution of poikilotherms—we have studied the same frequencies in *Cyprinus carpio* L. in relation to temperature. It appeared soon that the breathing rate in this fish is highly dependent on size. As a first contribution to the problem we intended to solve, this paper reports the influence of age and size on the mentioned physiologic trait.

Material and Methods

Our experiments have been performed on *Cyprinus carpio,* belonging to the race of glass-carps from the temperate warm waters. The best temperature for development for *Cyprinus carpio* would be situated, according to Huet (1953), between 20° and 25° C.; according to Schaeperclaus (1949) the optimum is nearer to 27° C. but at 15° C. a good production is still obtained.

As experimental objects we used fishes of three months, one year, two years and four years old, having live weights from 20 to 2000 grams. The fishes have been obtained from a single commercial stock.

A starvation period of at least 48 hours preceded all experiments.

Each individual of the different weight and age classes has been examined at several temperatures, from 4° C. up until death occurred, at about 38° C. Transfers always took place from lower to higher temperatures. Intervals can be read from the graphs to be discussed presently.

During measurements each temperature was kept constant within 0.1°, and the water abundantly aërated to provide a constant saturation with oxygen.

For practical purposes the fishes were put in small wire baskets. Measurements have been performed only on perfectly quiet animals.

Results

The dependence of the frequency of respiration upon the temperature in a single individual

1. Temperature acclimation

By temperature acclimation we mean the temporary adaptive alteration of the phenotype after an external temperature change.[1] It is known from Wells (1935a)

[1] We wish to reserve the term *adaptation* for a similar alteration of the genotype, which is included in the definition of *acclimation* or *acclimatization* by Prosser (1955) and by Bullock (1955).

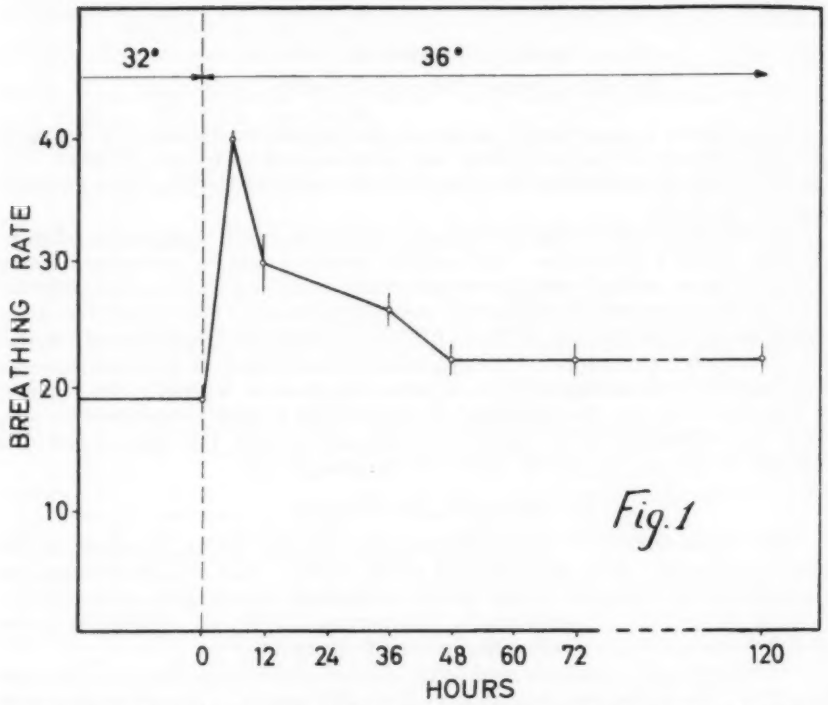

FIGURE 1. Successive mean respiration frequencies after transfer from 32° to 36°, plotted against acclimation time (carp No. 16). Vertical bars added to the points cover the range $M \pm \sigma$.

TABLE I

Frequency of respiration as a function of temperature
(Carp No. II)

Temperature (° C.)	Duration of respiratory pauses	Number of pauses per minute	Breathing frequency
4	1–2 mins.	1	2
8	½–2 mins.	2–1	6
12	15 secs.	3	9
18	—	—	24
15	16 secs.	3	13
20	10 secs.	5	24
26	10 secs.	5	25
29	10 secs.	5	24
34	10 secs.	5	24
35	—	—	24
37.4	3 secs.	15	32

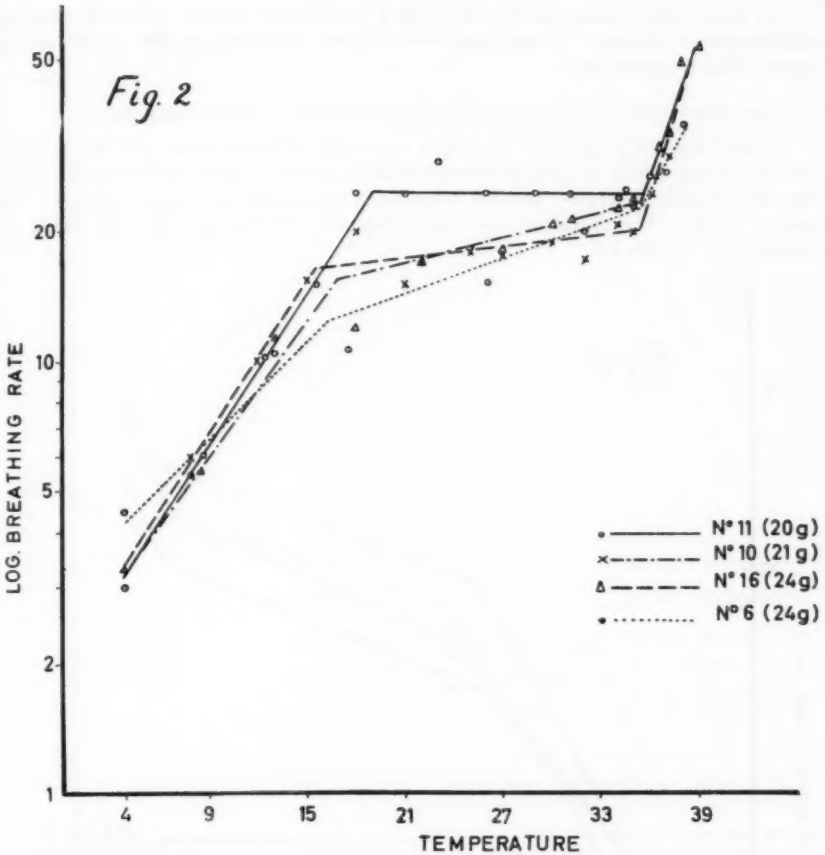

FIGURE 2. Rate–temperature curves for breathing frequency in carps of 20 to 24 grams.

and Schlieper (1950), that, in fishes, this acclimation takes from 24 hours to a few days after the transfer from one temperature to another. Only after this lapse of time, constant and consistent results, on metabolism at least, have been obtained. Before acclimation is completed, the oxygen consumption is generally higher than normal.

Figure 1 shows that similar phenomena characterize the accommodation of the frequency of respiratory movements. This frequency, after an initial rise of 100%, reaches a stable value after 48 hours following a transfer from 32° to 36° C. Usually a steady-state is reached within three to four days, although rarely it may last fourteen days. Acclimation is generally shortest between 16° and 30°. All fishes which maintained this steady-state for 48 hours were considered as completely acclimated. Only measurements obtained on such fishes are considered further in this paper.

At given high temperatures the fishes die before having attained complete acclimation as defined. These temperatures are indicated in this paper as the upper lethal temperatures.

2. Respiratory pauses and frequency of respiration at a given temperature

Typical for the respiration of fishes, especially of small ones, are the so-called pauses, during which no respiratory movements occur. After such a pause, which may be variable in length, the fish breathes a few times at a rapid rate and then pauses again. The duration and the frequency of the pauses vary with the temperature (Table I).

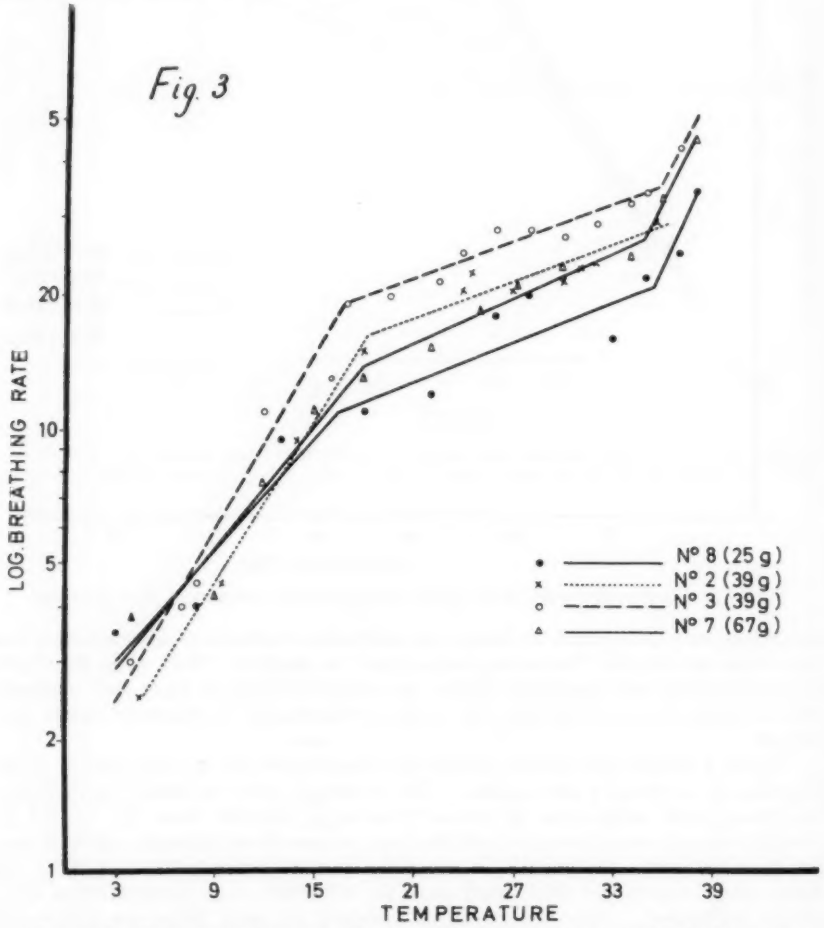

FIGURE 3. Rate–temperature curves for breathing frequency in carps of 25 to 67 grams.

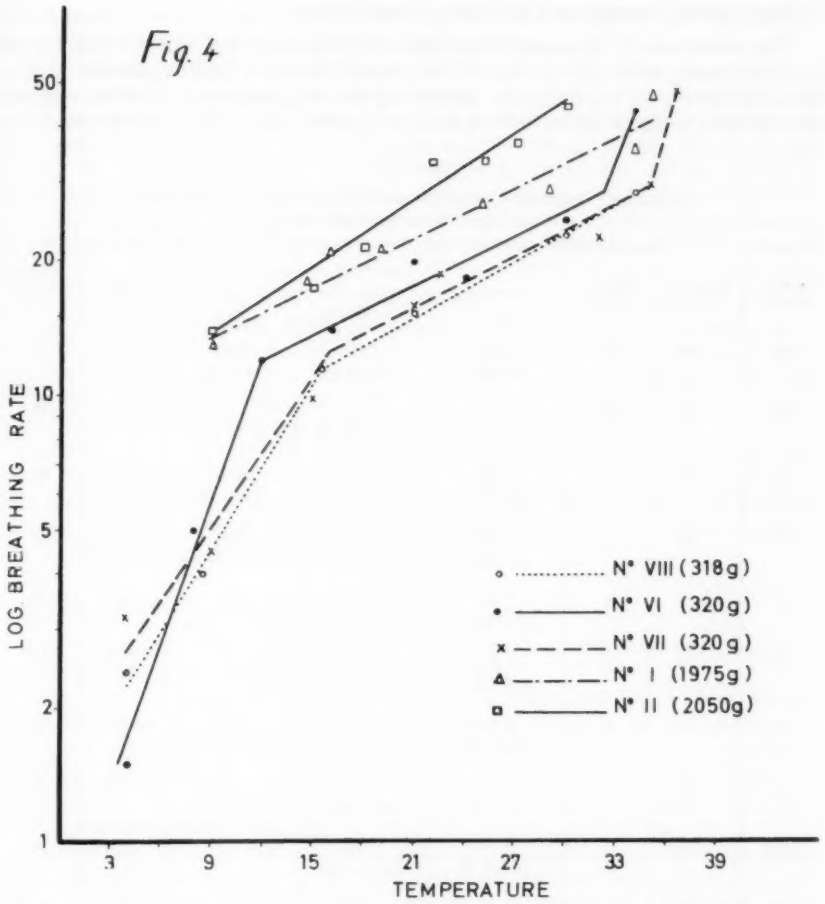

FIGURE 4. Rate-temperature curves for breathing frequency in carps of 318 to 2050 grams.

In this paper breathing frequency is expressed as movements per minute, ignoring the pauses. The time intervals used for one count were chosen long enough to eliminate the disturbing influence of the pauses. As a rule these intervals were five minutes at low, and two minutes at high temperatures, the counts being repeated at each temperature as many times as needed for adequate statistical treatment. Immediately after transfer to a higher temperature no respiratory pauses may occur, making for lower standard deviations (Fig. 1). Otherwise no trends in standard deviations could be detected during acclimation, with respect to time, or after acclimation, with respect to temperature.

3. Respiration frequency as a function of temperature

The variations of the mean frequencies of respiratory movements with respect to temperature, show, in the case of the smaller fishes, a typical pattern: first, at lower temperatures, a progressive increase of the frequency with a rise in temperature; further a less steep or even horizontal central part, where the frequency in-

TABLE II

Q_{10}-values, breathing rates at 24° and lethal temperatures for carps, arranged according to weight and age*

Serial number	Weight in grams	Age in years	Q_{10}			Rate at 24°	Lethal temperature
			Low	Intermediate	High		
11	20	¼	2.10 (4–19)	1.00 (19–35.4)	2.82 (35.4–37)	24.6	38
10	21	⅓	2.13 (4–15.6)	1.10 (15.6–35.4)	3.84 (35.4–38.4)	18.0	38.5
6	24	⅓	1.98 (4–17)	1.22 (17–35.2)	3.36 (35.2–38)	18.2	39
16	24	⅓	1.69 (4–16.3)	1.30 (16.3–35.3)	2.28 (35.3–37.8)	15.9	38
8	25	⅓	1.84 (3–16.5)	1.31 (16.5–35.4)	2.31 (35.4–37.5)	14.2	38
2	39	½	2.07 (4.7–18.3)	1.26 (18.3–35)	—	19.5	39
3	39	½	2.16 (4.7–17)	1.31 (17–35.5)	2.66 (35.5–37)	14.0	39
7	67	2	1.89 (4–18)	1.37 (18–35)	2.66 (35–37.7)	17.6	38
VIII	318	2	2.19 (4–15.5)	1.40 (15.5–34)	—	17.4	36
VI	320	2	2.83 (3.5–12.3)	1.35 (12.3–32)	—	20.2	36
VII	320	2	2.08 (4–16)	1.34 (16–35)	4.20 (35–36.5)	18.2	37
IX	325	2	—	—	—	—	35.8
I	1975	4	—	1.39 (9–35)	—	25.9	36
II	2050	4	←	1.51 (9–30)	—	33.0	36

* The temperature limits, in degrees C., are indicated in brackets.

creases much less with temperature, and finally a sudden rise of the respiratory rate at a point which is near to the lethal temperature.

This pattern is particularly well recognizable when the data are plotted on semilog coordinates, showing the proportionate response of the breathing rate to the temperature (Figs. 2, 3 and 4). This proportionate response appears to show, over broad temperature ranges, practically constant values, which change abruptly to other such values, at given critical temperatures. From eye-fitted curves through the data for individual fishes Q_{10}-values for each of these ranges can be readily estimated, while their intersections localize, with a fair approximation, the critical temperatures. The lower critical temperatures appear to be situated between 12.3° and 19°, the higher between 32° and 34.5° (Table II). The lower critical temperatures delimit a first zone of high Q_{10}-values from 1.69 to 2.83. Within the range extending from the low to the high critical temperatures Q_{10}-values vary from 1.00 to 1.51. Within the range of high temperatures Q_{10}'s reach values between 2.28 and 4.20. Because of the short interval separating the high critical and the lethal temperature, only a few data can be obtained in this range, making for a limited significance of these last Q_{10}'s for most fishes.

Nevertheless it is perfectly clear that for the population of carps studied, typical regulation phenomena characterize the respiratory rates over given homeostatic zones between 12.3° and 34.5°. However, within these zones a considerable individual variation, as to the Q_{10}'s, as well as to the situation of the critical temperatures, is showing up.

a. The influence of weight

A closer inspection of the log R-T curves in connection with the weight variants, leads readily to the recognition of the following facts.

First, all small fishes (39 grams and lower) exhibit more effective regulatory mechanisms than larger fishes. In the first category all Q_{10}'s, within the homeostatic zone, fall between 1.00 and 1.31, and, moreover, the correlation between weight and the respective values is very close (Table II). All larger fishes, on the other hand, have Q_{10}'s between 1.34 and 1.51 and, again, the largest fishes have very low regulatory capacities.

Concomitant with the occurrence of lower Q_{10}'s within the homeostatic zones of larger fishes, is the lowering of the upper lethal temperature threshold of the same fishes, with respect to the small fishes. Fishes of 67 grams and less have lethal temperatures at 38° and 39°, while those of higher weights have lethal temperatures between 35.5° and 37° On the other hand, the largest fishes (± 2000 grams) fall easily into a lethargic state at 4°, where the measurement of barely recognizable respiratory movements is no more possible. Therefore, a subdivision of the R-T curves of large fishes into three, with differential regulatory characteristics, seems to be impossible. Their narrowed viable range of temperatures can be characterized only by a single, high Q_{10}-value.

A last remarkable feature is the variation of the mean respiratory rate. The different individual R-T curves are clearly situated at unequal over-all rate levels. It is difficult to express this shift of the curves along the ordinates. We have tried to do this by indicating the estimated rate for all fishes at 24° (Table II). It appears, then, that the largest fishes have very high rates, smaller fishes attaining

only about half of these rates. The smallest fish, however, shows again a high over-all rate.

b. The influence of age

On the basis of our data it is not possible to differentiate clearly the effect of age from the influence of size. A certain indication is obtained from fish No. 7 (Table II). This fish was intentionally starved during one year in the laboratory. During that year its weight practically remained constant (67 grams), and was far below the weight of normally fed fishes of the same age (± 320 grams). For as far as the lethal temperature is concerned (38°) this fish reacted according to its weight class. Its Q_{10} within the homeostatic zone, however, falls within the range of its age group (1.37).

c. Residual variation

The number of individuals studied within each weight and age class does, of course, not allow an estimation of the individual variation of breathing rate within a homogeneous group. From our data we can, however, infer its existence. Most interesting, in this respect, is the fact that the R-T curve of fish No. VI is, in its whole, shifted to lower temperatures, for a distance of about 3°, when compared to fish No. VII, belonging to the same weight and age group (Fig. 4 and Table II). This might indicate a differential adaptation to temperature of both fishes.

Individual variation, with respect to the efficiency of the breathing regulation within homeostatic zones of equal extension, seems to exist as well. This is indicated by the detected differences in Q_{10}'s within age and weight classes.

Discussion

Two main points of interest are revealed by our analysis. The first is the shift of the upper lethal temperatures from 38°–39° for small fishes, to 35–36° C. for large-sized individuals. The second is the gradual disappearance of homeostasis for breathing frequencies with increase in size.

Decreased resistance to high temperature with size, if we restrict the survey to post-embryonic life, is not a general trend among fishes. The literature concerning the subject is reviewed by Hart (1952). All three types of fish species seem to exist: those with increasing, those with stationary, and those with decreasing resistance with increasing size. In most cases the separate effects of size and age have not been studied. In *Salvelinus fontinalis* no size effect was recorded for yearlings of the same age (Fry *et al.*, 1946), indicating seemingly that the age effect on temperature resistance would be preponderant. However, Spaas (unpublished results) found a size effect in one-year-old trout and a mean increase in high temperature resistance in two- and three-year-old fishes of the same species, which, however was lower than expected on the basis of the size differences for temperature tolerance within yearlings. Both factors, age and size, seem to interact in some way. The few data recorded in this study are not opposed to such a view.

A second main point, disclosed by our analysis, is the progressive increase of dependence of the frequency of respiratory movements upon the temperature. It

is illustrated by the gradual disappearance of a homeostatic zone of practical stability of this frequency at several temperatures in small fishes, and by a correlated increase of the over-all Q_{10}-value of this process in the course of life history.

By reputation, poikilotherms are animals without homeostatic mechanisms regulating their most essential physiologic functions, such as oxygen consumption, heat production, activity and development. More specifically, for oxygen consumption, they have been generally assumed to follow Krogh's "Normalkurve."

However, a certain number of workers have shown that the type of response to temperature of such functions is not only a specific trait among poikilotherms, but even that it varies intraspecifically as well.

According to recent reviews of the data (Rao and Bullock, 1954; Bullock, 1955), it seems justified to admit that in most cases a higher dependence on temperature is characteristic for animals from warmer habitats. A survey of the data on speed of development leads to the same conclusion with regard to specific and to racial geographic variation (Heuts, 1956). More effective homeostatic mechanisms seem to be a distinctive trait of inhabitants of colder areas, especially when intraspecific comparisons are made. Poikilotherms are poikilostatic organisms, but they are such to varying, genetically determined, degrees.

On the other hand, it is clear enough that the homeostatic mechanisms of the homoiotherms also are operating only within a given temperature range in a given individual (the thermoneutral zone), and, further, that the ranges of this zone are equally subject to specific variation in relation to the habitat, northern animals possessing more efficient homeostatic mechanisms operating at low temperatures (Scholander *et al.*, 1950).

The conclusion is obvious, and has been drawn, that a homeotherm is clearly adapted to the temperature range where the Q_{10} for biological processes appears to be equal to 1.00, and where, consequently, its behavior is most homeothermic.

In poikilotherms such homeostatic zones, with Q_{10}'s approaching 1.00, are generally not found or not recognized.

Only recently Bullock (1955) strongly called attention to the existence of homeostatic mechanisms for a number of rate processes among several poikilotherms. In addition to the cases cited by this author a few other examples may be mentioned here.

A clearly delimited homeostatic zone for oxygen consumption is indicated, although not recognized by the author, in *Fundulus parvipinnis* between 18° and 22° for small fishes (Wells, 1935a, 1935b). Its existence shows up due to almost continuously increasing experimental temperatures between 10° and 24°. A definite fall of Q_{10} values for oxygen consumption between 15° and 20° in *Salvelinus fontinalis* is demonstrated by Job's data (1955). Unfortunately no measurements have been made beyond 20°. The same author mentions the results of Edwards (1946) on the click beetle *Melanotus communis,* which, between 17° and 27°, shows a definite decrease of Q_{10}-values for oxygen uptake. Job, however, is inclined to regard this phenomenon as due to an experimental error.

A number of indications seem thus to point to a possibly widespread phenomenon of homeostatic mechanisms among poikilotherms, which may be brought to light by more accurate experimental procedures. These zones being presumably narrow, their absence in current observations might be due to the experimental

procedure making use of too discontinuous temperature gradients. Still another source of error might be an insufficiently long acclimation to the experimental temperatures.

If, however, homeostatic zones can be demonstrated to exist in poikilotherms, then it seems logical to admit, as is done in homeotherms, that they delimit adaptive zones, and to conclude that a poikilotherm is adapted to such conditions, where it is least poikilostatic. Such a situation exists clearly in young carps. In the smallest fishes Q_{10}-values approach 1.00 over a temperature range from 16° to 35°. Within this range they are homeostatic for the studied trait, and to this range they are probably best adapted.

How far the frequencies of respiratory movements reflect the oxygen consumptions at different temperatures remains, of course, an open question. They will not truly reflect oxygen consumption, if, principally, the utilization coefficient of the oxygen present in the water, flowing over the gills during each breathing movement, would be different according to temperature. For trout and eel, at least, van Dam (1938) has shown that the utilization percentage is not affected by a temperature difference of 8°.

For obvious reasons, the complete disappearance of homeostatic zones in carps, with increasing size, cannot be asserted. They certainly are narrowed in the course of life, though this progressive alteration is not, or not directly, dependent on age. A similar narrowing of homeostatic zones is obvious in *Fundulus parvipinnis* (Wells, 1935b), concomitant with the over-all higher dependence of oxygen uptake on temperature with increasing size.

In other cases, however, the individual evolution is exactly the opposite. Job (1955) recognizes a flattened proportionate response to temperature, a higher temperature independence, in large-sized *Salvelinus fontinalis*. Similarly Spaas (unpublished results) finds an increased dependence of oxygen consumption in large versus small yearlings of brook- and sea-trout.

The click beetles already mentioned (Edwards, 1946) show, on the other hand, a response of oxygen uptake to temperature per unit wet weight, which is independent of size. The case is, however, not strictly comparable, because all click beetles, whatever their dimensions, have reached final sizes.

Between the first fact disclosed by our analysis (the shift of upper lethal temperature to lower values in the course of life) and the second (the decrease in homeostatic efficiency) there seems to be a close relation in our material, as one would logically expect. Lack of adequate data on post-embryonic development prevents the generalization of this observation for individual life cycles. Some data on embryonic development, however, indicate clearly that a similar relationship does not hold in racial and interspecific comparisons.

Races or species with a higher temperature independence for speed of embryonic development can nevertheless have a narrower temperature tolerance range during development than another race or species with a more dependent development. If the relationship between temperature tolerance limits and regulation holds in individual cycles, then this cycle can be labelled as physiologically regressive (with respect to the general trend of the phylogenetic record) in *Cyprinus carpio* and in *Fundulus parvipinnis*. Several Salmonidae seem to follow a physiologically progressive ontogeny.

A last point of interest is the fact that the methods used seem to be adequate to

detect individual adaptive differences to temperature ranges, as well as individual differential degrees of homeostatic attributes, without necessarily killing the experimental objects. This permits genetic studies of the mentioned characteristics, and creates thus the possibility of filling gradually the evolutionary gap between poikilotherms and homeotherms.

Summary

1. The dependence of the frequencies of respiratory movements upon temperature has been studied in *Cyprinus carpio* L.

2. The degree of dependence of these frequencies upon temperatures is primarily determined by the size of the fishes. Large fishes are highly, small fishes only slightly, dependent.

3. Small fishes are characterized by a broad homeostatic zone of independence of the breathing frequency on temperatures. This homeostatic zone disappears in large fishes. The upper lethal temperatures decrease concomitantly with the disappearance of the homeostatic zones.

4. Individual variants, as to the degree of homeostasis and as to the adaptive temperature ranges, can be detected by the method presented. It can be used for studying genetic determination of these characteristics.

5. A survey of the data in the literature allows one to distinguish poikilotherms with progressive and with regressive ontogenies, with respect to homeostatic behavior.

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OXYGEN UPTAKE IN INSECTS WITH CYCLIC CO_2 RELEASE

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The periodical release of carbon dioxide (CO_2), as first found by one of us in bugs (Punt, 1944), is well established now in several insects in rest (Punt, 1950, 1956a) and in diapausing pupae of Lepidoptera (Punt, 1950; Schneiderman and Williams, 1953, 1955; Buck, Keister and Specht, 1953; Buck and Keister, 1955).

As to the oxygen uptake, there is some controversy among the above mentioned authors. Punt described in *Carabus nemoralis* (Müll.), *Locusta migratoria migratorioides* (Rch. and Frm.), *Meloë proscarabaeus* L. and *Rhodnius prolixus* (Stål.) a diaferometrically recorded periodical oxygen uptake, which ran parallel to the periodical CO_2 bursts.

In diapausing pupae, on the other hand, a continuous oxygen uptake was found by Schneiderman and Williams and by Buck and Keister using the Warburg technique.

The aim of this paper is to give more details about our investigations concerning the oxygen uptake in carabids and in the pupae of the cecropia silkworm. Some results of experiments on the influence of oxygen tension on the burst frequency will be mentioned. A possible explanation of the oxygen records will be given and the preliminary results of the estimation of the CO_2-binding power of insect haemolymph will be discussed.

Material and Methods

The insects under investigation were *Carabus nemoralis* (Müll.), *Hadrocarabus problematicus* Hrbst., *Periplaneta americana* L., pupae of the cecropia silkworm (*Hyalophora cecropia* (L.)) and pupae of *Sphinx ligustri* L.

The cecropia pupae were received from Dr. Schneiderman to whom we want to express our acknowledgment here.

A single animal was put in a glass tube, through which a very constant current of CO_2-free outdoor air was sucked. The gas exchange was continuously estimated by means of two diaferometers as described earlier (Punt, 1950, 1956a). In these instruments the gas which is to be analyzed passes through a copper tube, in the center of which an electrically heated platina wire is suspended. The temperature of the wire, and as a consequence the electric resistance, depends on the CO_2 and O_2 percentage, respectively, of the passing gas. The wire is connected in a Wheatstone bridge with a galvanometer. In all experiments two parallel diaferometers were used to record the CO_2 production and the O_2 uptake simultaneously. The gas coming from the animal container was dried and equally divided among both machines; the portion for the O_2 diaferometer was absolutely freed from CO_2 by soda lime. As the O_2 reading is slightly influenced by the removal of the CO_2, a valid R. Q. cannot be extracted directly from the curves. Both galvanometer

responses were recorded on one paper strip in a rotating-drum camera, the slit of which was perpendicular to the direction of rotation. Care was taken that the diaferometers had the same "latent period" in order to get synchronous points on the ordinate of the graphs. The galvanometers were connected in such a way that O_2 uptake and CO_2 release were recorded in the same direction.

In other experiments, for comparison, the direct Warburg method was used to estimate the O_2 uptake. The animals were placed in standard Warburg flasks (16 ml.) containing filter paper drenched in 10% KOH. The temperature was kept constant at 20° C.

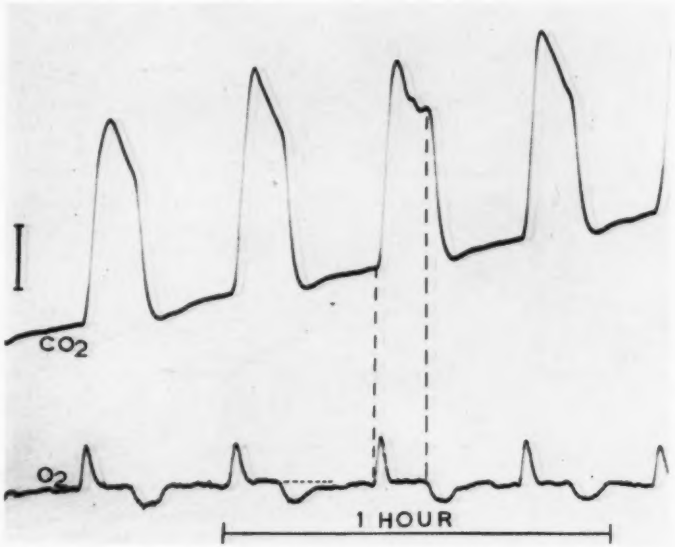

FIGURE 1. Photographically recorded CO_2 production and O_2 uptake of one specimen of *Carabus nemoralis*. The index on the left side indicates approximately 10 μL per hour (O_2 and CO_2). The inclination of the CO_2 line is caused by galvanometer drift; 20° C.

The CO_2 dissociation curve of haemolymph was estimated by means of the Haldane method for blood gas analysis. In order to work with small quantities, a special apparatus was built with a 10-ml. reaction vessel, the thermo-barometer vessel being of the same size. Gas mixtures were analyzed in the Haldane gas analysis apparatus.

RESULTS

1. Oxygen uptake in carabids

In a previous paper (Punt, 1956a) the discontinuous oxygen uptake in *Carabus nemoralis* (Müll.), *Locusta migratoria migratorioides* (Rch. and Frm.), *Meloë proscarabaeus* L. and *Rhodnius prolixus* (Stål.) was described.

The oxygen uptake was found to be (at any rate partly) periodical; the spikes in the photographic records were exactly synchronous with the CO_2 bursts. But

there could be noticed a marked difference between the forms of the O_2 and CO_2 curves, which difference was most evident in *Carabus* (Figs. 1 and 2).

Both the opening and the closing moments of the spiracles are rather distinct in the CO_2 line: a steep curving up of the galvanometer record indicates the moment of opening and a more or less sharp notch in the line, followed by a decline to zero level (or so close to zero that the difference was within the range of experimental error), marks the moment of closing. Sometimes, in *Carabus* and *Periplaneta*, the line rises somewhat in the interburst period (Fig. 1), probably due to small interburst CO_2 release (*cf.* Schneiderman and Williams, 1955).

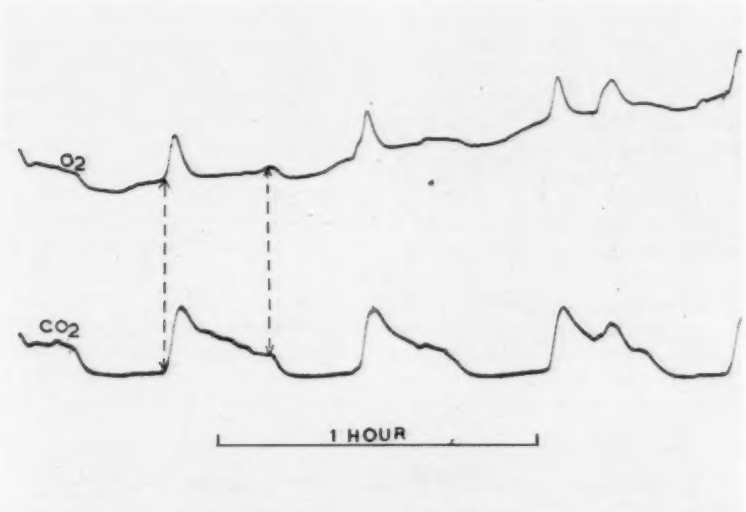

FIGURE 2. As Figure 1, but from a beetle in which the ratio between the open period and the closed period is discrepant from the mean value (1:2) as is occasionally found.

The O_2 line, too, shows these peculiarities, but while the CO_2 spike shows a maximum just after opening of the spiracle and declines rather regularly till the moment of closing, the O_2 line goes down much steeper nearly to the interburst level. After the moment of closing of the spiracles (corresponding to the sharp bend in the CO_2 line) the O_2 line declines still more to a minimum, to curve upwards again after some time to come to a "steady-state" which is maintained till the next burst. This steady rate of oxygen consumption is slightly lower than the minimum O_2 uptake in the burst period. As at that time we only meant to determine the type of respiratory activity and not the total gas exchange, there were unfortunately no zero readings recorded in the above mentioned paper. The respiration was recorded over periods of 6 hours continuously and it is rather inaccurate to interpolate a zero line over so long a period, particularly as there may have been some galvanometer drift due to external circumstances.

Next we performed some experiments of much shorter duration, preceded and followed by zero readings in order to be able to interpolate the zero line in our

records. Though there was considerable individual variation, the O_2 uptake in the interburst periods could be calculated from planimeter measurements to be approximately 60% of the total O_2 consumption. So in *Carabus* the oxygen uptake is both continuous and periodical. In a great number of experiments with *Periplaneta americana* we came to the same conclusion. It has already been shown that these O_2 spikes could not be caused by CO_2 interference in the O_2 diaferometer (Punt, 1956a).

As was mentioned, Schneiderman and Williams, and Buck and Keister reported the O_2 uptake in diapausing pupae to be continuous only. In order to determine whether this discrepancy between our results in *Carabus* and the results of Schneiderman and Buck was the consequence of the use of different techniques, the gas ex-

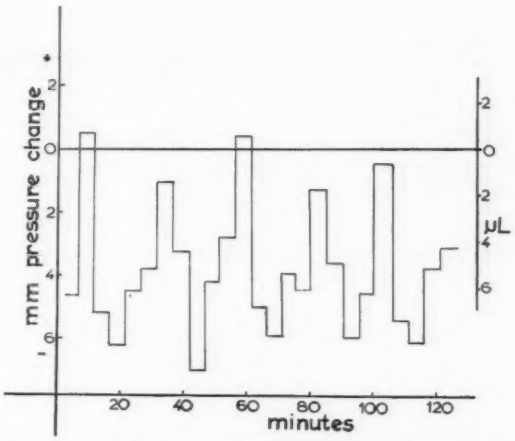

FIGURE 3. Manometrically estimated O_2 uptake in *Hadrocarabus problematicus* (440 mg.). Warburg flasks provided with KOH-drenched paper; 20° C.

change of carabid beetles was estimated in a Warburg apparatus. There were performed a great number of experiments with specimens of *Hadrocarabus problematicus* of a weight of 250–650 mg. The insects were placed in a normal side-arm flask with gas vent. The side-arm contained filter-paper, which protruded into the flask above the beetle. The paper was drenched in 10% KOH. After half an hour of temperature equilibration the stopcocks were closed and manometer readings made at intervals of three or five minutes. The change in pressure in this interval as compared with the foregoing reading was plotted against time (Fig. 3). In this way the oxygen consumption could be calculated from these figures, taking the flask constant into account. As the insect's volume was not exactly known, the index on the right of Figure 3 represents an arbitrary value only. The results show that O_2 consumption in *Hadrocarabus* is at any rate partly periodical, which is in accordance with the diaferometer experiments. Sometimes the curve rises above the zero line, which would indicate that there is in this interval an increase in pressure in comparison with the foregoing reading. This can only mean that at these mo-

ments the CO_2 bursts occur and the CO_2 absorption is not yet completed. But as the CO_2 is readily absorbed, the pressure is considerably decreased in the following five minutes, accentuating the apparent O_2 uptake in this period. From a large number of experiments with the diaferometer we know that at this temperature (20° C.) in carabids the open spiracle period can be sharply distinguished from the closed period, the interburst period being mostly twice as long as the burst. As this ratio cannot be clearly seen in the manometrical curves it may be concluded that in *Hadrocarabus* O_2 uptake is partly continuous as well.

When instead of containing KOH-drenched filter paper the side-arms were empty, the line represented in Figure 4 was found. Here, too, the moment of opening of the spiracles could be found in the curve as an increase in pressure,

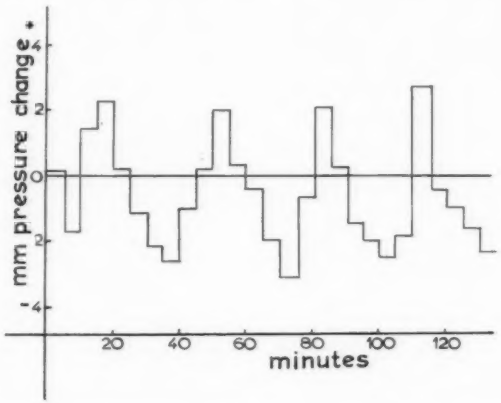

FIGURE 4. *Hadrocarabus problematicus* (500 mg.). Variations in pressure in a Warburg flask, without KOH; 20° C.

reaching a maximum in about ten minutes. This was followed by a slow decrease in pressure, lasting for about fifteen to twenty minutes by which the cycle was completed.

The decrease in pressure in the closed period is interpreted as a continuous O_2 consumption, but Buck, Keister and Specht (1953) considered the possibility that telescoping of the insect was involved, which in this manometric method could not be distinguished from some other phenomenon causing pressure loss. Our results, however, in the "open circuit" with the diaferometer made this assumption doubtful. Still we thought that in this beetle the space between the abdominal tergites and the elytra, which can be rather well closed and into which eight out of nine pairs of spiracles open, had something to do with the cyclic gas exchange. So we perforated the elytra. This had not the slightest influence on the periodical respiration. The sealing of three pairs of the spiracles with melted bee's wax made the periods more irregular and increased their frequency. It did not matter very much which pairs of spiracles were sealed. Introduction into the spiracles of very tiny glass capillaries, which were fixed with bee's wax, made the O_2 uptake more con-

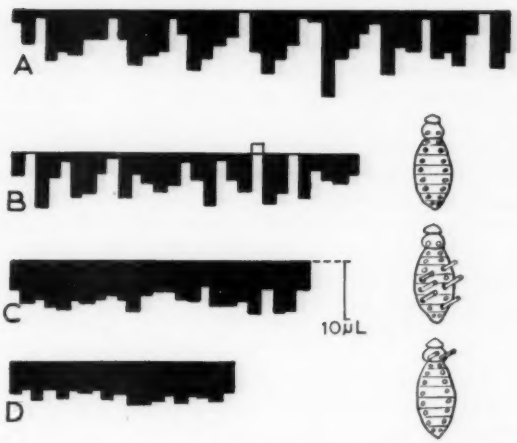

FIGURE 5. Oxygen uptake in *Hadrocarabus*, manometrically estimated at three-minute intervals; 20° C. A: normal insect at rest, the elytra being removed. B: Some of the spiracles sealed with bee's wax. C: Some of the abdominal spiracles kept open with glass tubes. D: The same with the thoracic spiracles.

tinuous. The clearest effect resulted here from placing the capillaries in the large thoracic spiracles (Fig. 5).

2. The oxygen uptake of Hyalophora cecropia pupae

Two years ago, in the Physiological Laboratory, University of Utrecht, the following experiments were performed with cecropia pupae. The gas exchange was

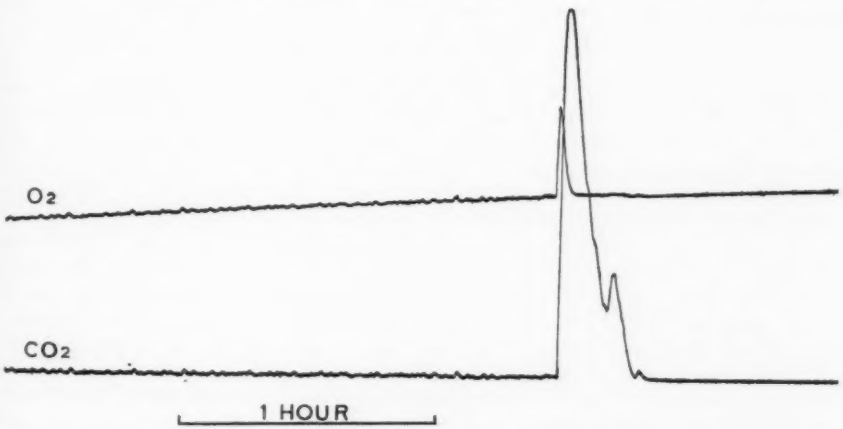

FIGURE 6. Photographically recorded CO_2 release and O_2 uptake in a pupa of the cecropia silkworm; 20° C.

estimated with two diaferometers simultaneously, one for recording the CO_2 production, the other for the O_2 consumption. In Figure 6 the photographically recorded galvanometer responses are shown. At 20° C. these diapausing pupae showed one CO_2 burst in about four hours. The form of the CO_2 line is exactly the same as described earlier in pupae of *Sphinx ligustri.* Just after the CO_2 spike the line falls to approximately zero level and is very smooth, but after about one hour small perturbations return in the line, which go on and increase until the next CO_2 burst. Exactly synchronous with the CO_2 bursts small O_2 spikes were found. This O_2 line falls after some minutes but remains slightly above the interburst level. In the interburst period the O_2 line, too, is at first very smooth, but shows after some time the same small perturbations as the CO_2 line (in fact running exactly parallel to them). It looks as if the spiracles are no longer hermetically closed but are leaking or fluttering from time to time. Unfortunately we did not esti-

FIGURE 7. The same pupa as in Figure 6. A double burst is recorded.

mate the exact value of the O_2 uptake in these prolonged experiments, as was pointed out in the foregoing section. But as the O_2 diaferometer was adjusted to be approximately as sensitive as the CO_2 apparatus in order to get the same response for the same percentage of gas, it is clear from these graphs that O_2 uptake must take place in the interburst periods as well. The area of the O_2 spike was much smaller than that of the CO_2 burst, whereas the interburst perturbations were of the same size in both lines. The impression which we have from some zero readings of the O_2 line and from calculations of the O_2 spike area is that there must be a relatively large interburst O_2 uptake which was considered to have a mean value of 92% of the total O_2 uptake. This confirms the findings of Schneiderman and Williams and of Buck *et al.* But still a periodic O_2 uptake is found as well. Perhaps of interest in this connection is what we find on page 147 in the paper of Buck and Keister (1955) where we can read: "Small but statistically significant perturbations were in fact seen in some of our O_2 uptake records. . . ."

Occasionally a double burst was recorded as was already described for pupae of *Sphinx* and *Papilio* (Punt, 1950; Fig. 7).

FIGURE 8. Gas exchange of a cecropia pupa, a few weeks before hatching (May, 1954). Drawn from photographic records.

In Figure 8 we find a record of simultaneously estimated CO_2 release and O_2 uptake of the same cecropia pupae made a few weeks before hatching. The graph is on the same amplification and on the same time scale as Figure 6. The burst frequency has increased to two per hour. The O_2 uptake in the bursts is relatively larger.

3. Influence of oxygen tension on burst frequency

The influence of CO_2 tension on the burst frequencies in *Carabus* has been previously described (Punt, 1955, 1956b). Increasing pCO_2 caused a prolonged open-

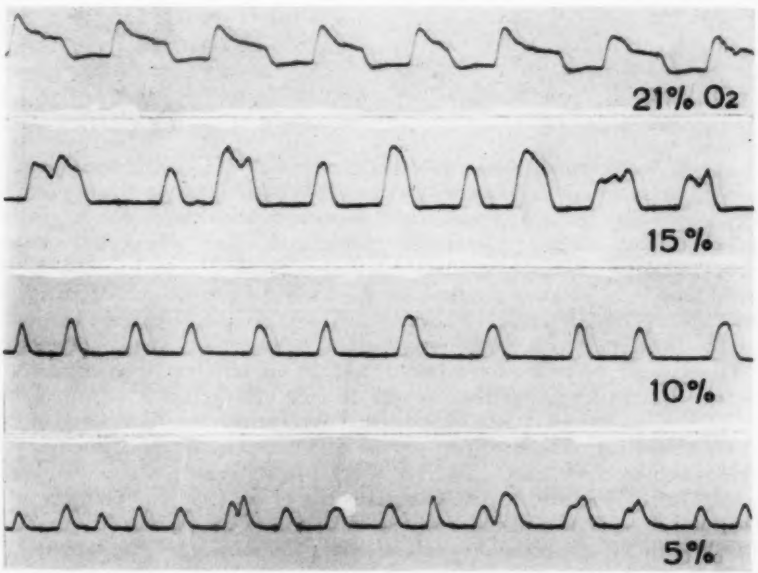

FIGURE 9. *Hadrocarabus problematicus;* CO_2 burst frequency as influenced by different O_2 tensions.

ing of the spiracles, until at about 1½% of CO_2 the closing of the spiracles was inhibited. In the present investigation experiments were performed on the influence of decreasing oxygen tension on burst frequency in *Hadrocarabus problematicus*. In Figure 9 a summary is given of the results. A decrease of pO_2 caused an increase in CO_2 burst frequency and consequently a decrease in burst volume. This is in accord with Wigglesworth's observation on spiracular movement in fleas (Wigglesworth, 1953).

4. *The CO_2 dissociation curve of haemolymph*

For reasons given in the discussion, we thought it worthwhile to know something about the CO_2 dissociation curve of insect haemolymph. Much work has been done on the composition of the body fluid of insects, but as to the possibility of buffering the CO_2 our knowledge is scanty (Reali, 1955; van Asperen and van Esch, 1956; Levenbook and Clark, 1950). We therefore tried to estimate the CO_2

TABLE I

The CO_2-binding property of haemolymph of Sphinx ligustri pupae

Quantity of haemolymph (mL)	pCO_2 (mm.Hg)	Total CO_2 (volume %)
0.50	0	2.2 ± 0.8
0.70	0	1.7 ± 0.6
0.69	0	2.2 ± 0.6
0.49	19	5.1 ± 0.9
0.23	31	10.9 ± 1.0
0.50	38	9.2 ± 1.1
0.53	38	8.9 ± 0.9
0.38	73	18.2 ± 1.4
0.24	84	17.9 ± 2.2
0.33	105	20.0 ± 1.5

saturation of haemolymph under different tensions of CO_2. The haemolymph of diapausing pupae of *Sphinx ligustri* was gathered by puncturing the pupae with a syringe which was previously moistened with tromboliquine (heparin), in order to prevent clotting. The exactly measured quantity of haemolymph was put in the small bulb-flask of the Haldane blood-gas analyzer and saturated with CO_2 under a certain tension by rotating the flask mechanically for one hour. After that time the analysis was performed in the normal way, tartaric acid being used to expel the CO_2 from the haemolymph. The results are to be found in Table I and in Figure 10. The data are corrected for dissolved CO_2 at the different tensions and at the temperature used (20° C.) so that the total amount of CO_2 could be plotted against the pCO_2. Though only a few experiments were performed (our stock of pupae being exhausted for this season), it proved that the CO_2-binding capacity exceeds the line which represents the dissolving of CO_2 in water at the same temperature (the solid line in Figure 10; data from Umbreit *et al.*, 1949). Our data are too few in number to allow drawing a correct line through the points.

Insect haemolymph probably contains some CO_2-binding principle but in our pupae the capacity was rather disappointing as only twice as much CO_2 could be extracted from the haemolymph as would dissolve in pure water. In certain other

insects (*Gastrophilus* and *Hydrophilus; cf.* Florkin, 1937) the quantity of bound CO_2 was found to be much larger, but these may perhaps represent special cases. More work on this subject is in progress.

Discussion

We will try to give a possible interpretation of the recorded O_2 uptake and CO_2 release in *Hadrocarabus*. This interpretation is, of course, only hypothetical, but

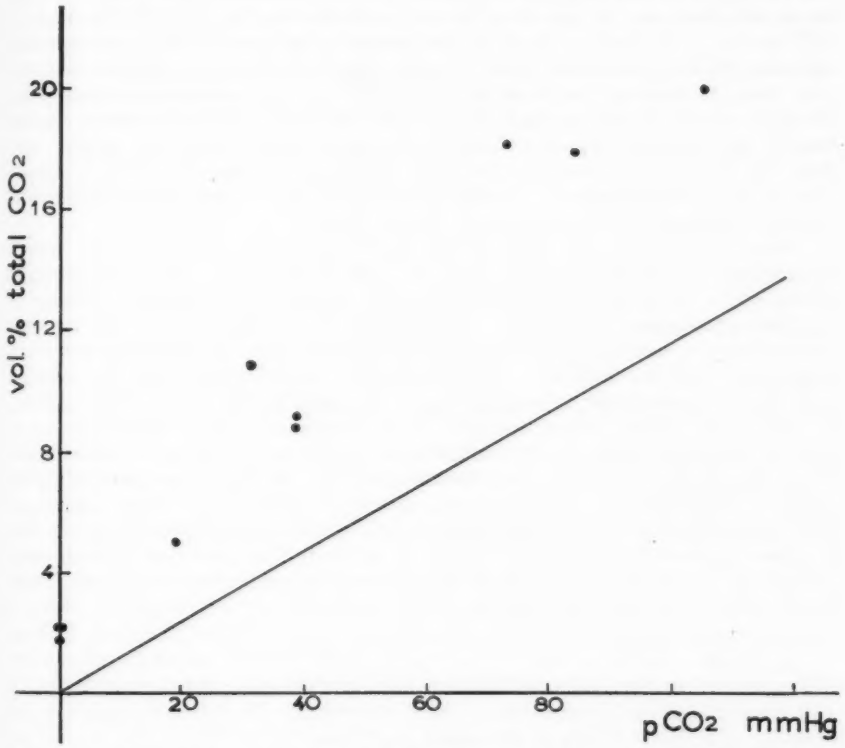

Figure 10. The CO_2 capacity of haemolymph of *Sphinx ligustri* (20° C.). Solid line: CO_2 capacity of pure water.

is in agreement with the results of our and other investigations and partly based upon assumptions from Wigglesworth (1953) and from Buck and Keister (1955, p. 162). Probably in other insects, including diapausing pupae, the same phenomena may occur.

We may assume that oxygen is taken up continuously in some way or another. But the quantity which can penetrate into the insect body in the "closed spiracles"

period is insufficient to cover the metabolic oxygen want. So an oxygen debt is incurred, oxidation being incomplete, and acid metabolites are formed and accumulated in the body fluid. This is in accordance with the theory of Wigglesworth, who attributes the withdrawal of fluid from the tracheal endings to increased osmotic value of the tissue fluids, due to the increasing amount of acid metabolites.

Carbon dioxide is formed by metabolic processes and stored in the body fluid, not only as dissolved gaseous CO_2 but probably bound in some buffer system or CO_2-binding principle. But as soon as the pO_2 in the tracheal system is diminished and an oxygen debt develops in the tissues, the acid metabolites drive the CO_2 from the bound phase into the gas phase and as a consequence the pCO_2 in the tracheae will increase. As soon as the pCO_2 has reached a certain threshold the spiracles open and the CO_2 burst takes place. In the open period the CO_2 diffuses out, not only from the tracheae but from the tissue fluid as well, until the moment when the pCO_2 reaches a level at which the spiracles may close. In the meantime O_2 has entered the tracheae, which is seen as the initial spike in the O_2 uptake line. But after this initial spike the O_2 line falls down to a level slightly above the steady state of the interburst period. Probably this part of the O_2 line represents the real oxygen consumption of the animal per unit of time.

When the spiracles are closed at last, the amount of O_2 in the tracheal system is sufficient to cover the metabolic want for some time (the O_2 line going down to nearly zero), but as soon as the pO_2 inside the trachea is low enough, a leakage of O_2 starts (continuous O_2 uptake). This leakage is not sufficient, however, to cover the real oxygen want and acid metabolites are formed again, by which the described respiratory cycle is completed. This hypothesis is in accordance with the assumption that CO_2 controls the sudden opening of the spiracles (Buck and Keister, 1955; p. 161). The action of decreased pO_2 on the triggering of the spiracular opening may be seen as an indirect one: decreased pO_2 increases the degree of hypoxia and hence the amount of gaseous CO_2 liberated into the tracheae. Similarly Wigglesworth's assumption that in pure oxygen, opening is induced by a large amount of CO_2, and in 5% O_2 by a very small amount of CO_2, could be interpreted to mean that in pure oxygen the CO_2 remains bound in the body fluid and that in both cases (100% of O_2, and 5% of O_2) the tension of free CO_2, triggering the spiracles, may be the same. Probably this threshold CO_2 tension is not very high, for there is evidence that in about 1½% of CO_2 the spiracles do not close at all (Punt, 1956b).

So it looks as if in all cases it is the pCO_2 which controls spiracular movement. When oxygen tension is lowered, oxygen debt develops more readily and the pCO_2 will reach the critical level sooner. As a consequence the burst frequency is increased. The burst volume is decreased, as no large quantities of CO_2 can be accumulated in the short interburst period. It would be worthwhile to determine the pH of the haemolymph under these circumstances. When oxygen tension is raised, acid metabolites are not formed so soon and more metabolic CO_2 may be bound in the body fluid, the intertracheal pCO_2 only reaching the critical value after a longer period; burst frequency is decreased, burst volume increased (Punt, 1956b). As to the experiments in pure oxygen, when the spiracles are opened the tracheae are filled with pure oxygen and as a consequence the oxygen leak in interburst period is much less. Schneiderman and Williams (1955; p. 134) stated that the "interburst CO_2 output," too, may become undetectable in pure oxygen.

Summary

1. The simultaneous determination of CO_2 production and O_2 uptake in *Hadrocarabus problematicus* Hrbst. is described. Both CO_2 release and O_2 uptake are cyclic, but there is some interburst O_2 uptake.

2. The diaferometrically recorded O_2 uptake is discussed, and compared with the results as obtained with the Warburg technique.

3. In diapausing pupae of the cecropia silkworm (*Hyalophora cecropia*) the continuous O_2 uptake forms a larger percentage of the total amount of O_2 consumption than in *Hadrocarabus*. Still there was found an initial maximum of O_2 uptake of short duration at every CO_2 burst.

4. Some experiments on the CO_2 dissociation curve of haemolymph of pupae of *Sphinx ligustri* are mentioned. There is evidence that in haemolymph a CO_2-binding principle is present.

5. The interaction of O_2, haemolymph and CO_2, resulting in a certain intertracheal pCO_2 controlling spiracle movement, is discussed. Probably the pO_2 is only indirectly involved in the triggering of spiracle opening.

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STUDIES ON MARINE BRYOZOA. X. HIPPADENELLA CARSONAE, N. SP.

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The purpose of this paper is to give a detailed description of the morphological features of *Hippadenella carsonae*, new species and to note other species which are ecologically associated with it.

Hippadenella carsonae is a lepralioid Ectoproct, Order Cheilostomata, Suborder Ascophora, of the Family Hippoporinidae of Bassler (1953). It is named in memory of Louisa Carson, a beloved teacher of my early school years.

Species Data

Hippadenella carsonae, n. sp.

Diagnosis: Colony calcareous, twig-like; branching open. Twigs slender, cylindrical, with usually nine to twelve, sometimes more, wedge-shaped zooecia in cross-section. Mural rims raised, crinkled. The smooth to tubercled frontal is a pleurocyst with three to five pairs of oblique, tubular pores. Orifice lepralioid, rounded. Lyrula absent but two slight cardelles are placed low. Operculum with lateral parenthesis-like sclerites. Very broadly oval median suboral avicularium placed at a varying angle to the frontal plane and mounted on a wide porous avicularial chamber that contains prominent avicularial glands, muscles and vestigial polypide. Hyperstomial ovicell very salient, globose, but slightly flattened, tubercled and non-porous. Its arched rim is a continuation of the zooecial mural rims. Two communication areas (a multiporous pore chamber and corresponding opening) in lateral wall. Distal wall a sieve plate of numerous pores.

Measurements: The first figures are the minimum, the next the maximum and the last (in parentheses) the average of ten readings (or occasionally more) for each structure. Readings are in millimeters. L is for length, W for width, D for diameter, H for height.

1.073–1.406	(1.247)	L	Zooecia
0.407–0.555	(0.444)	W	Zooecia
0.115–0.173	(0.146)	L	Avicularial apertures (rostal and back areas)

0.101–0.173	(0.137)	W	Avicularial aperture
0.058–0.086	(0.072)	L	Mandible
0.094–0.173	(0.124)	W	Mandible
0.144–0.202	(0.173)	L	Zooecial orifice
0.144–0.216	(0.179)	W	Zooecial orifice
0.158–0.187	(0.170)	L	Operculum
0.173–0.202	(0.192)	W	Operculum
0.432–0.475	(0.452)	L	Ovicell
0.389–0.490	(0.422)	W	Ovicell
0.675–0.705	(0.690)	L	Tentacles (two readings only)

The zooecial polypide measurements below are based on only one reading each:

0.147	D	Tentacular bundle within the tentacular sheath
0.264	L	Esophagus
0.132	D	Esophagus, just below the tentacles
0.073	D	Esophagus, just above the cardia
0.029	H	Esophageal epithelial cells near tentacles
0.014	H	Esophageal epithelial cells near cardia
0.132	D	Stomach caecum (empty)
0.250	L	Rectum (empty)
0.088	D	Rectum (empty)

Zoarium: The zoarium or colony consists of openly branching twigs which sometimes fuse or anastomose with other twigs at point of contact. The twigs are fragile and brittle, breaking into shorter twigs, so that no true picture of the extent of a colony, nor the ultimate pattern of branching, whether dichotomous or irregular (Figs. 11, 13) can be gotten from the mass of short fragments at hand. Some of the broken fragments were up to 51 mm. long and about 2 or 3 mm. in diameter. There was about a pint of material in the collection, but not one of the stalks was a complete, intact colony.

To the naked eye the twigs appear smoothly cylindrical (Figs. 13, 21) except in the ovicelligerous region where they are covered with bumps (ovicells) as in Figure 11. The usual number of zoids around a branch is about nine to twelve, although occasional stalks may have almost twice that number just before a bifurcation (Figs. 14, 21). Zoids face outward around the usually cylindrical branches radially.

Dead twigs are white, living twigs yellow. In practically all the living twigs, *i.e.*, twigs collected when they were in the living state, the polypides were confined to the tips or distal part of the stalk while the basal, proximal part of the twig was dead, polypideless. The yellow color of live twigs is due to the presence of yellow polypides within the zooecia.

Zooecia: The outside calcareous skeletal case in which the soft zoid parts (digestive tract, musculature, tentacles) are housed is the zooecium. Zooecia are wedge-shaped in cross section (Fig. 21), mostly rectangular in frontal or face view and quincuncially arranged (Figs. 6, 12) like bricks in a wall. In side view the end wall (Figs. 8, 10) slants a bit obliquely downward and backward. In some zooecia

it is nearly horizontal. The frontal wall is of variable thickness, sometimes being twice as thick as the side wall.

Zooecial boundaries are well defined by mural rims (Figs. 3, 12, 17, 18, 23). The height of these partitions varies with age and with exposure to molar forces. Sometimes these rims are high and very crinkled. Other times they are nearly level with the zooecial front. In early secondary calcification the distal mural rims send slender calcified trabeculae across the operculum (Figs. 18, 23). Later, the orifice may be incompletely obliterated by more extensive calcification over the operculum (Figs. 6, 17).

The frontal wall is flat to convex, porcellanous to sparsely tubercled, non-porous centrally but completely perforated by about three to five pairs, usually three pairs (Fig. 10), of obliquely directed marginal tubular pores or tubes whose diameter varies (Figs. 30, 32). These tubes end in slight elliptical craters above the frontal (Fig. 32). Viewed from the front, the top pair of tubes slants diagonally, converging downward toward the zooecial mid front. The middle pair slants toward the zooecial mid line. The bottom pair slants diagonally upward toward the zooecial mid front, as in Figure 30. In other words, the marginal tubes lean toward the zooecial center front.

The front may be perfectly smooth except for the raised pores or it may be roughened by a few tubercles (Figs. 6, 17).

The side walls are straight and have two widely spaced, multiporous, blister-like interzooecial communication areas. These areas are variously known in bryozoan literature as rosette plates, septula, pore chambers, corresponding openings. The distal one is a pore chamber and the proximal one is a corresponding opening (Figs. 8, 10, 19, 26). The pore chambers have six to twelve small pores (Fig. 19). The corresponding opening has a single large hole rimmed about by an irregular annulus (Fig. 26). The quincuncial arrangement of zooecia brings the pore chambers of one vertical row of zooecia against the corresponding openings of the left or right vertical rows of neighboring zooecia, and vice versa. Silén (1944) has given an excellent and extensive account of pore chambers, corresponding openings, pore plates and various types of interzooecial communications for various species. Conditions in *Hippadenella carsonae* are in agreement with his findings for related species.

The end wall is oval to pear-shaped (Figs. 10, 21). Its smaller, medial part is punctured by numerous (about forty, more or less) small, closely spaced pores and slants downward more than the broader peripheral non-porous part. Sometimes the slant is rather steep, sometimes deviating little from the horizontal.

The calcareous frontal walls are a bit too opaque usually for satisfactory study of soft internal parts as tentacles, gut, gonads, musculature and avicularial apparatus. Decalcification was not attempted because of the nature of the colony—a number of zooecia radially arranged, very close together. Crushing the stalk gently in a drop of Euparal on a slide usually gave well dispersed material quickly and in reasonably satisfactory condition for microscopic study. Attempts were made to clear the youngest, slenderest, polypide-containing tips in glycerine and others in dioxan. Some clearing was accomplished with the glycerine but none with the dioxan. So, the description of the soft internal parts is based on crushed, dispersed material and also on what could be seen through the walls of the younger, less calcified zooecia.

For a study of the exoskeleton or zooecial walls calcining (burning off the chitinous and membranous coverings with a small blow pipe) is the most satisfactory method of preparation. However, calcining must be halted before the specimen becomes too fragile and disintegrates to powder.

Autozooecial polypide: The autozooecial polypide consists of the tentacles, gut and associated musculature (Figs. 2, 7, 16, 29). All polypides are in a retracted position, the polypides withdrawn into the body cavity, none with tentacles extruded through the orifice, so characteristics of the retractor muscles, parietal body wall muscles and tentacle number could not be studied. As far as can be deduced from retracted specimens there seem to be about twelve to fourteen tentacles. The tentacles are ciliated, rather stout and not of excessive length. They are withdrawn into a transparent membranous tentacle sheath which is closed at the top near the operculum (Fig. 16) by a sphincter or diaphragm. Two flask-shaped "oral" or "sub-oral" glands of unknown function are part of the sheath, just beneath the diaphragm. Attached to the diaphragm are two bundles of muscle fibers, the parieto-diaphragmatics, one on each side (Figs. 2, 16, 29). Their muscle fibers extend diagonally back and upward to attach to the zooecial wall. A short distance below the parieto-diaphragmatics are two membranous bands, the parieto-vaginals, which contain a few delicate fibers that are deviated from the tentacle sheath. The parieto-vaginals connect the tentacular sheath to the lateral zooecial walls.

The gut consists of mouth, esophagus, stomach (which has three divisions: cardiac, caecal and pyloric), rectum and anus. In some bryozoa there is a ciliated pharynx between mouth and esophagus but not in *H. carsonae*. The esophagus of *H. carsonae* is short and tapers slightly. Its epithelial mucosa cells are tall columnar, hyaline and not ciliated. Those near the mouth are twice as tall as those near the cardia, the diminution in height being gradual. A sphincter separates the esophagus from the cardia. Retractor muscles attach the lophophore (region at the base of the tentacles and around the mouth) to the body wall, so there originates a curtain of muscle fibers just at the beginning of the esophagus. The epithelial cells of the cardia are low, cuboidal, non-ciliated and not much different in diameter

List of Abbreviations Used on the Plates

A Abductor mandibuli muscle fibers
B Adductor mandibuli muscles
C Avicularial back area
D Avicularial chamber
E Avicularial gland
F Avicularial polypide
G Avicularial rostral area
H Avicularium
I Cardelle
J Cardia
K Esophagus
L Mandible
M Mural rim
N "Oral" gland
O Orifice or aperture
P Ovicell
Q Parieto-diaphragmaticus muscle
R Parieto-vaginal band
S Pore chamber or rosette plate
T Pylorus
U Rectum
V Sclerite
W Stomach
X Tentacles
Y Tentacular sheath

All figures, except Figures 4, 8, 10, 11 and 13, were drawn with the aid of a camera lucida, and are of *Hippadenella carsonae*, new species, from Antarctic type locality, Sta. 104. Figure 11 is from the holotype, the others from paratypes. Measurements for structures are given in text.

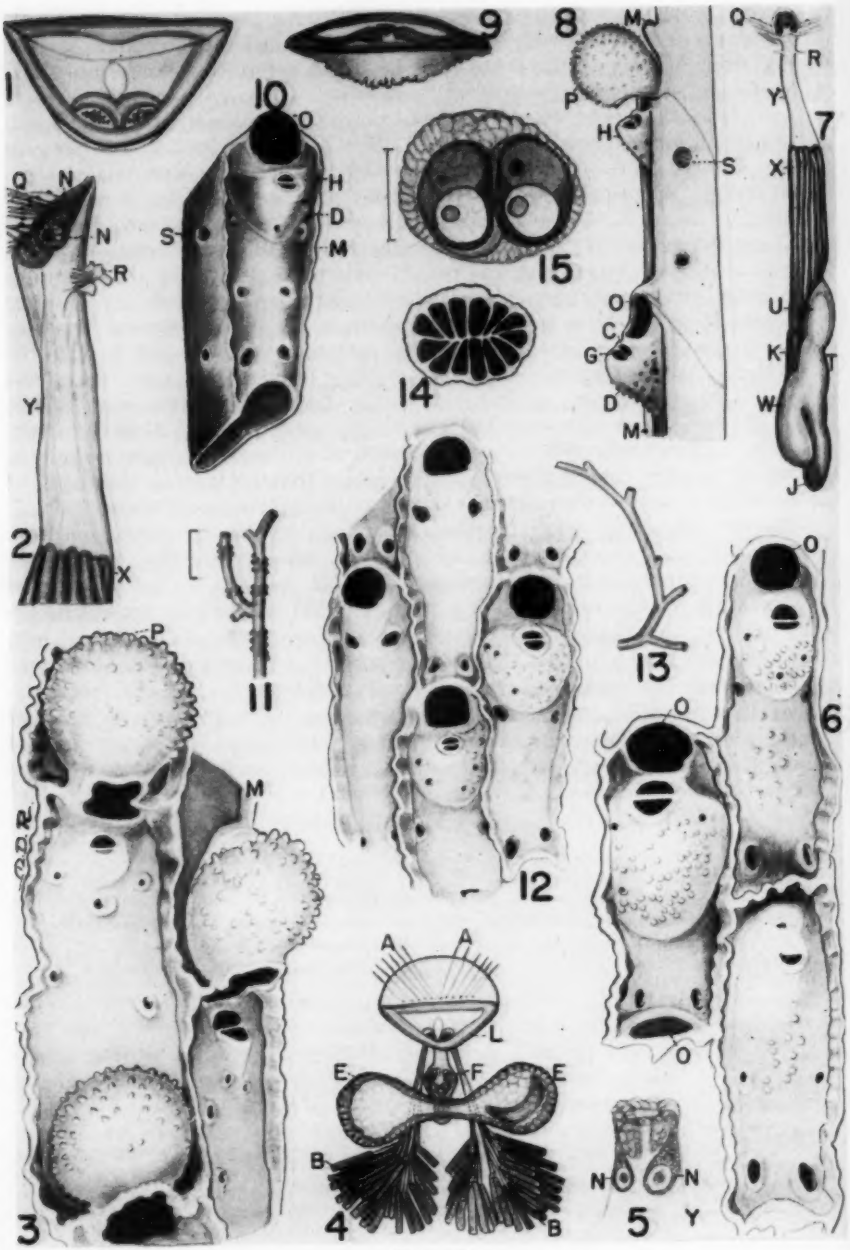
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PLATE I

from those of the esophagus but their cytoplasm is granular rather than hyaline. The cardial wall is thin. The cardia is J-shaped, parallelling the esophagus. The stomach caecum originates at the upper side of the cardia, near the pylorus, and has a highly granular cytoplasm. The pylorus is ciliated, internally. The different parts of the stomach and also the rectum are all rather thin-walled. Diatoms must be a part of the *H. carsonae* diet because diatom shells are in some of the recta.

The gut and tentacles amply fill the body cavity.

The ovary is attached to the side wall, behind the tentacular sheath and below the operculum. It is present in non-ovicelled zoids and presumably also in ovicelled ones as well as in zoids with or without an avicularium. If an avicularium is

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EXPLANATION OF PLATE I

FIGURE 1. Mandible, top surface view. Transparent oval in center is the lucida. Adductor muscle fibers attach as individual dots in the two semicircular areas in front of lucida. Transparent rimming membrane (*cf.* Figs. 9, 25) here barely visible about rounded tip.

FIGURE 2. Side view of upper third of polypide. Compare with Figures 7, 16, 29.

FIGURE 3. Frontal view of three calcareous ovicelled zooecia. The upper two have avicularia whose chambers are small. From calcined specimen.

FIGURE 4. Diagram showing contents of avicularial chamber, as seen from top and front. One of the avicularial glands contains a hardened or coagulated crescent-shaped secretion. The delicate abductor muscle fibers are shown bent upward, out of their natural position, for the sake of clarity. They should extend downward and backward toward the base of the avicularial chamber, some distance away from and back of the adductor tendons.

FIGURE 5. Side view of "diaphragm" or sphincter and the two "oral" glands which contain some secretion. The sphincter is slightly relaxed, so is evident a narrow central passageway that must widen considerably to permit extrusion of tentacles.

FIGURE 6. Frontal view of three calcareous zooecia. The upper two have fully developed and functional orifices and avicularia. The bottom one has a nearly obliterated orifice and avicularium due to secondary calcification. Avicularial chambers are large as compared with those of Figure 3.

FIGURE 7. Retracted polypide. The slender upper third corresponds to Figure 2 but is from a different side. The esophagus is partly hidden by the stomach caecum. The anus is at the uppermost tip of rectum.

FIGURE 8. Diagrammatic side view of zooecia, front wall to the left, side wall facing observer. Ovicelled zooecium complete, the non-ovicelled one only partly shown. The end walls slant, sometimes less obliquely than shown, may even be nearly horizontal in some zooecia. Side walls are perforated by two communication areas. Upper, distal multiporous area (S) is like an internal blister (*cf.* Fig. 19) and is called a rosette plate or pore chamber. Lower, proximal single opening fits against a rosette plate of a neighboring zooecium which is not here shown. Single opening bears the inadequate name of "corresponding opening."

FIGURE 9. Mandible, edge view. The delicate serrated membrane hangs vertically down from front edge. More heavily cuticularized parts are darkened.

FIGURE 10. Diagram of a single, non-ovicelled, wedge-shaped zooecium. Its side wall (with two communication areas) is at left. Its frontal wall (with six frontal pores) is at right. A multiporous end wall is at bottom. Three pores are shown on the avicularial chamber.

FIGURE 11. Colony fragment, drawn to the one-cm. scale at immediate left. Bumps along stalk are ovicells.

FIGURE 12. Frontal view of four zooecia, two with avicularia and two without. From a calcined specimen.

FIGURE 13. Another colony, with more branches. Drawn to same scale as Figure 11.

FIGURE 14. Cross-section of a slightly flattened branch which has thirteen wedge-shaped zooecia at this level.

FIGURE 15. Four eggs in ovary. Nucleoli prominent, excentric. Nuclei clear, vesicular. Cytoplasm homogeneous and denser. Drawn to the 0.06-mm. scale at left.

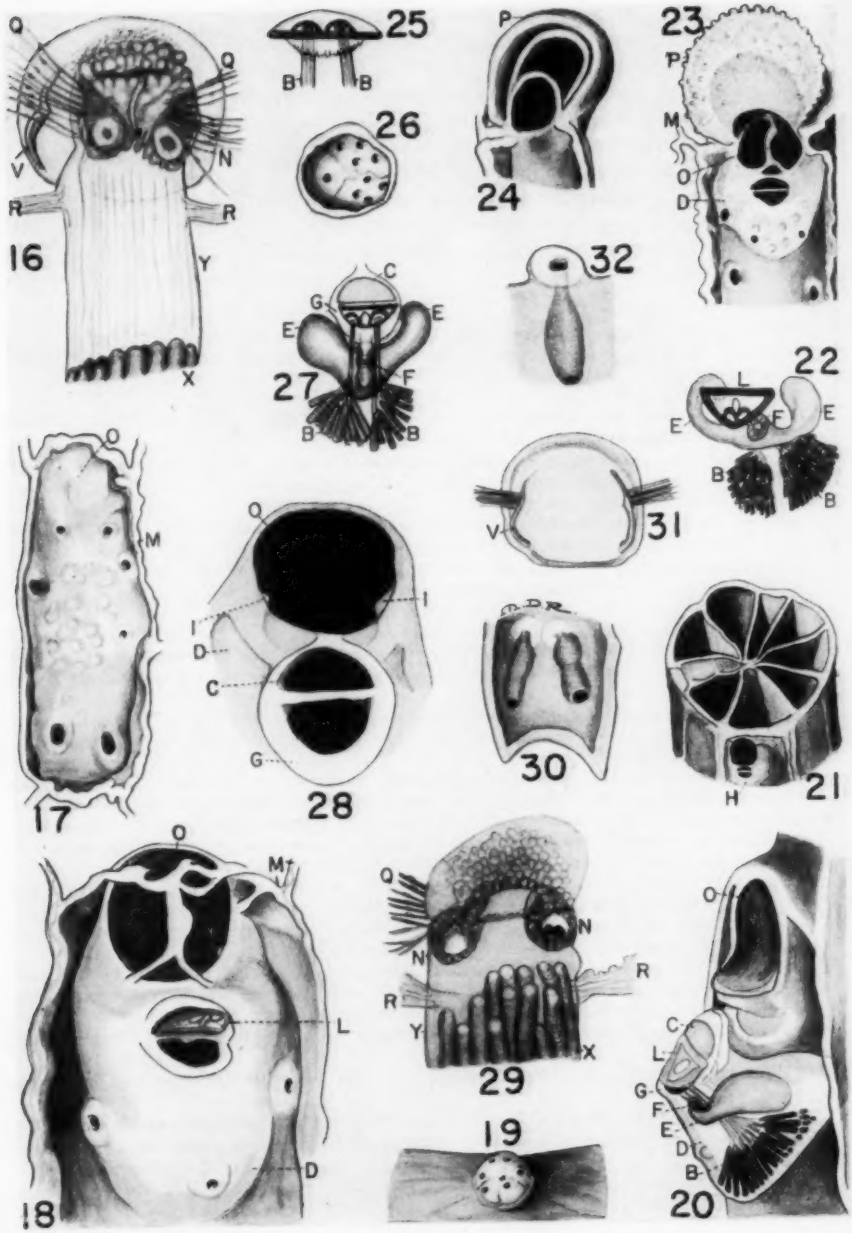
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PLATE II

present the ovary is about half-way between the operculum and the avicularial chamber, back of the tentacular sheath. It contains several eggs of different sizes. The ova have a large excentric nucleolus and an almost clear nucleus (Fig. 15). Whether an ovary is present in every zoid cannot be ascertained because of the opacity of zooecial walls. Whether this species is hermaphroditic or dioecious is unknown at present. It was not possible to determine the exact site of origin of spermatogenic tissue although some material, apparently spermatogenic, was found near the stomach in rather sizable patches in the crushed specimens.

Orifice: The *H. carsonae* orifice resembles those of the genera *Cryptosula* and *Hippodiplosia*. It is rounded, lepralioid. Part of its distal wall is formed by the next distal zoid. A median tooth, lyrula, is absent. Two slight cardelles or ledges for operculum articulation are placed low at the sides, dividing the orifice into two areas of unequal size. The larger distal area is formed by the anter (distal orifice lip) while the smaller proximal area is bounded by the poster (proximal orifice

EXPLANATION OF PLATE II

FIGURE 16. Operculum, "diaphragm," "oral" glands, tentacle sheath, tentacle tips and anchoring musculature shown from the back surface. The channel for protrusion of tentacles is more contracted than in Figure 5.

FIGURE 17. Frontal view of zooecium with completely sealed-over orifice in a more advanced stage of calcification than that of Figure 6.

FIGURE 18. Frontal view of the upper half of a zooecium in an early stage of secondary calcification where calcareous trabeculae extend from the mural rims across the orifice. A partly opened mandible articulates with the pivot.

FIGURE 19. Multiporous rosette plate of side wall.

FIGURE 20. Profile of zooecial orifice and avicularium with operculum and mandible, respectively, in place. The avicularial wall is left more transparent than it normally would appear to show the position of the avicularial contents. Compare with Figures 4, 22, 27.

FIGURE 21. Stereogram of typical cylindrical stalk. Orifice and avicularium on bottom zooecium. Porous end wall tops another.

FIGURE 22. Oblique top view of avicularial apparatus. Glands curve around adductor tendons to fit the avicularial chamber.

FIGURE 23. Front of a zooecium which has an ovicell and avicularium, and shows secondary calcification trabeculae spanning the orifice. Ovicell opening is completely plugged up by domed calcareous lamina which occupies about half the ovicell interior and which is here faintly outlined, *cf.* Figure 24.

FIGURE 24. Ovicell whose front wall has been broken away to show the double outer wall and the third (innermost) lamina which has sealed off the ovicell cavity. The lamina resulted from secondary calcification.

FIGURE 25. Edge view of another mandible with serrate membrane and adductor muscle tendons which attach to semicircular areas (*cf.* Fig. 1).

FIGURE 26. Looking through a "corresponding opening" with its irregular annulus or ledge, into the multiporous blister-like rosette plate (*cf.* Fig. 19).

FIGURE 27. Avicularial contents from the under side. Abductors are not shown.

FIGURE 28. Detail of orifice, cardelles and avicularium. The bands leading from orifice to avicularium represent differences in thickness of frontal calcification and are also the distal limits of the avicularial chamber.

FIGURE 29. Frontal view of "oral" glands, operculum and tentacle tips which are very close to extrusion. The "diaphragm" opening is wider than in Figures 5 and 16.

FIGURE 30. Interior of frontal zooecial wall showing shape and direction of tubular frontal pores. The interior openings have been blackened. The exterior openings are faintly rimmed to show their raised nature (*cf.* Figs. 3, 6, 32).

FIGURE 31. Inner face of operculum. Occlusor muscles attach to sclerites.

FIGURE 32. Side view of frontal wall tubular pore and its raised external terminus.

lip). The cardelles form the dividing line between the two areas, *cf.* Figure 28. In side view (Fig. 20), the orifice is not a flat plane. The poster curves outward in lepralioid fashion. There is no difference in size between orifices of the ovicelled and non-ovicelled zooecia.

The zooecial orifice is covered by an operculum shaped to fit (Figs. 20, 31).

It has been the universal and long-time practice for bryozoologists to use the term "chitinous" when describing opercula of those bryozoa which have a more or less horny, yellowish to brown, sometimes unevenly stiffened or reinforced operculum. Its use is based more on the visible physical appearance of the substance (its resemblance to the insect exoskeleton) rather than on its chemical composition. Dr. Libbie Hyman suggests that the term "cuticularized" would be more appropriate since little chemical evidence exists for the presence of chitin in the ectoprocts (bryozoa). So, where I have used the term "chitinized" in the present and in past articles in connection with bryozoa the term "cuticularized" would perhaps have been more suitable.

The *H. carsonae* operculum is lightly cuticularized (Fig. 31). Near its lateral borders, internally, are parenthetical sclerites to which attach delicate occlusor muscle fibers and from which a flange may develop in some older zoids.

The orifice in many old zoids is overgrown or secondarily calcified and may be entirely sealed over or obliterated (Figs. 17, 18). Sometimes the secondary calcification extends to the avicularium and ovicell, so that the avicularium too fuses over and the ovicell opening is blocked by a dome-shaped calcareous lamina (Figs. 6, 23, 24). Peristome and oral spines are absent.

Ovicells: The ovicells were collected long past the breeding season because larvae are absent. The ovicells are either empty or partitioned off by the internal secondary calcification lamina.

Ovicells generally occur in groups at periodic intervals along the stalk. They are not immersed or covered over by the frontal of the next zoid but rest on a cushion formed by it (Figs. 3, 8). They are large, salient, non-porous. Their surface is beaded to tuberculate and faintly ridged. The thin raised rim about the highly arched opening is continuous with the raised mural rims.

Avicularia: An avicularium occurs on some zoids, either ovicelled or non-ovicelled. Its size varies from small to medium, some avicularia being twice as large as others. In position it is constant, always sub-oral, median, slanting obliquely forward-downward, away from the orifice.

A pivot bar or hinge (Figs. 18, 28) for articulation with the mandible separates the avicularial surface into two slightly inclined regions: (a) the back area and (b) the mandibular, beak or rostral area. The rostral area is closed by the mandible. The membrane-covered back area is shorter and broader than the rostral area and in this species is always nearest the orifice, the rostral area being the farthest away, both in the mid line and sub-oral (Fig. 20).

In face view the avicularium is broadly oval, wider than long, perched on a wide mound-like avicularial chamber of variable size. Usually three, occasionally more or fewer, small pores perforate the front of this chamber.

There is a great difference in the degree of development or complexity of the soft structures inside the avicularia of various species but in *H. carsonae* they are well developed.

Levinsen (1909) called the avicularia and their contents heterozooecia and heterozooids; Borg (1926) heterozoids; Silén (1938) heterozoids, avicularial polypides, polymorphic individuals. Earlier references exist to the avicularial contents (Waters, 1888, 1892, 1900) but Waters' 1892 account is very adequate and understandably figured, although Silén (1938) published an extensive and excellent study of avicularia of several species.

The avicularial contents of *Hippadenella carsonae* are similar to those described by Waters (1892, pp. 272–274, and Pl. 19, Figs. 1, 2, 4, 5) for *Lepralia foliacea* Ellis and Sollander. In *H. carsonae* the avicularial chamber contains the avicularial polypide, avicularial glands, abductor mandibuli and adductor mandibuli muscles (Figs. 4, 20, 22, 27). The function of the polypide and glands is unknown. Marcus (1939, Pl. 19, Fig. 46) shows the avicularial contents of seven species and on p. 275 says of avicularial glands: "These glands can neither belong to the nervous, nor to the nutritive, or reproductive system and might perhaps have something to do with the stronger skeleton of the Ascophora . . . the function of the(se) organs still remains unknown; they might be poisonous." The *H. carsonae* avicularial glands are sometimes large, hollow and alveolar, with a large lumen and thin wall. Other times they are partly filled with a homogeneous, hardened secretion. The two bilaterally placed glands are united in a saddle-shaped unit that curves about the two bundles of adductor tendons and follows the contours of the very wide avicularial chamber. The avicularial polypide is a small, dense cellular body pinched in the middle so it seems double. It is attached to the back of the avicularial gland isthmus, in the mid-line. A vestibulum connects the polypide to the rostral area below the mandible tip, although this is difficult to see because of the opacity of the calcareous wall, the infrequency of favorably oriented crushed specimens and the small size of the soft structures involved.

The musculature of the avicularium is well developed. The adductor mandibuli muscles are more anterior, proximal and massive than the abductor mandibuli muscles. The adductor muscles have numerous short, thick, faintly striated muscle fibers. Their endings at origin and insertion differ in appearance. Their origin is over an extensive area on the walls and floor of the avicularial chamber. Their insertion is in two small pits, one at each side of the lucida on the inner mandibular surface. The muscle fibers attach bluntly and broadly at the origin while before the insertion is reached the muscle fibers have given way abruptly to delicate membranous and fine tendinous tissue (Figs. 4, 20). The tendon fibers attach by bead-like enlargements to the insertion site (Figs. 1, 25, 27).

The abductors form a very diffuse, sparsely fibered curtain against the distal end of the avicularial chamber. Their fibers originate on the back avicularial chamber wall and insert on the cuticularized membrane very close to the pivotal bar against which the mandible articulates. They do not seem to be striated and are considerably more slender than the adductor fibers. Marcus (1939, p. 273) states that in species studied by him the avicularial adductors are striated but the abductors smooth. This is true of *H. carsonae* also.

The avicularium is topped by a cuticularized mandible and a back area membrane (Figs. 20, 27). The mandible has a narrowly elliptical lucida flanked on each side by a somewhat semicircular pit into which the adductor tendons attach. A broad cuticularized band reinforces the mandible edges and base (Figs. 1, 22). A

short, delicate, transparent and serrated membrane decorates its front border (Figs. 9, 25), hanging down vertically, so it is almost invisible from the top (Fig. 1).

Distribution and ecology: Hippadenella carsonae turned up in only two dredgings of Jan. 29, 1948, Comdr. D. C. Nutt collector; one fragment from Sta. 101, and a pintful of twigs from Sta. 104. Both stations were from the Ross Sea area, Antarctica, off Cape Royds, Ross Island, from 58 fathoms. Most of the twigs were empty of polypides and relatively clean of extensive extraneous growths or encrustations. When other forms did grow on or in them these were relatively few in number and sparsely distributed over the branches, so apparently the twigs of *H. carsonae* did not present as hospitable a stratum for settling of other forms as does *Phylactellipora lyrulata* or some other species. The following organisms or their products are attached to the dead parts of *H. carsonae* twigs from Sta. 104: brown and green Folliculinids, Foraminifera, brown and white sponges, yellow egg cases (of flatworms?), calcareous tubes of annelids and scraps of various bryozoa. The bryozoa growing in small patches or attached to *H. carsonae* are several species of the Order Cyclostomata and the following of the Order Cheilostomata: *Cellaria moniliorata, Hippothoa bougainvillei, Hippothoa distans, Phylactellipora lyrulata* and a number of other species awaiting fuller identification. The Cyclostomata are especially numerous and seem to favor this species. Some Sta. 104 *H. carsonae* twigs grow on or partly engulf alcyonarian and sponge spicules, Cyclostomata, *Cellaria moniliorata, Cellaria vitrimuralis, Smittina ordinata* and other cheilostomes yet to be identified. In a discussion of *Smittina ordinata* (Rogick, 1956, p. 300) reference was made to *Smittina ordinata* growing on "other Bryozoa at Sta. 104," the other bryozoa in this case being *H. carsonae.*

Hippadenella carsonae specimens are deposited in the Smithsonian Institution, U. S. National Museum, USNM Cat. Nos. 11357 through 11364.

Summary

1. The morphology of *Hippadenella carsonae,* new ectoproct from the Antarctic, is described in detail and measurements made of many of its structures.

2. At the time of its collection, Jan. 29, ovicells were empty of embryos but developing eggs were in the ovaries. Developing embryos were absent from the body cavity also.

3. Living polypides occurred at the tips of some twigs but most of the material was dead or empty of polypides, at the time of collection.

4. The species has an unusually well developed avicularial apparatus consisting of large glands, reduced polypide, abductor and adductor muscles.

5. So-called "oral" or "sub-oral" glands are present. They are near the operculum but have no actual connection with, or proximity to, the true polypide mouth.

6. The function of "oral" glands, avicularial glands and avicularial polypide is unknown in this species, and a matter for speculation in other species.

7. Other peculiarities of this species are the oblique tubular frontal wall channels (frontal pores) and the mode of secondary calcification where trabeculae span the orifice and a domed lamina seals the ovicell.

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EXCYSTATION OF APOSTOME CILIATES IN RELATION TO MOLTING OF THEIR CRUSTACEAN HOSTS

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The dependence of certain aspects of the life cycle of a parasite on certain physiological activities of its host is a common phenomenon. One need only mention, as examples, the synchronicity of reproduction in malaria parasites, which is dependent on the diurnal rhythm of activity of the host (Stauber, 1939), and the appearance of sexual reproduction in the intestinal protozoa of the roach *Cryptocercus*, which is provoked by the molting of the host (Cleveland and Nutting, 1955). In a similar way, some species of apostome ciliates exist as small cysts (phoronts) on the gills of various Crustacea and excyst only at the time when the host molts (Chatton and Lwoff, 1935). The excysted forms (trophonts) then engorge rapidly on the proteinaceous fluid in the shed skin of the host. The much larger organisms so produced form free-living cysts. Within these cysts a series of divisions occurs which results in the formation of a number of small daughter ciliates (tomites). These swim about, apparently without feeding, until they have been drawn into the gill chamber of a suitable crustacean host. Here they encyst on the gills and again remain quiescent until the new host molts. The entire cycle depends on a single meal obtained from the molting fluids of the host, and the molting of the host provides the only stimulus to excystation (Chatton and Lwoff, 1935).

It seemed of interest to attempt to produce excystation *in vitro*.

Materials and Methods

Two host-parasite combinations have been used: (1) the fiddler crab *Uca pugnax* and a probably undescribed species of *Gymnodinioides;* (2) the hermit crab *Pagurus longicarpus* and *Gymnodinioides inkystans*. The fiddler crabs were kept in dishes with a shallow layer of sea water which was changed daily. Isolated individuals were kept in paper cups with a little sea water and a screen cover. The hermit crabs were maintained in running sea water. Isolated crabs of this species were placed in 100-ml. beakers covered with a piece of gauze held on by a rubber band. The beakers were immersed in an aquarium of running sea water. Both species were fed pieces of clam once or twice a week.

Individuals of *Uca pugnax* which were near the molt were generally recognizable by a peculiar pale cast to the carapace and legs. Those in the process of molting were observed to have milky white blood. In order to identify crabs very near the molt, the tip of a leg was cut off to permit a drop of blood to exude. The blood had a clear appearance except just before molting, when it was milky.

Crabs of the species *Pagurus longicarpus* near molting could be recognized by a marked gray color of the carapace and legs, as distinguished from the reddish cast

of individuals which had recently molted. In small groups of isolated "gray" crabs about 50% molted within three days after isolation, whereas none molted in corresponding groups of "red" crabs.

For the *in vitro* experiments, sterile Petri dishes holding two discs of filter paper and one or two depression slides were used. The filter paper was moistened with sterile sea water. In the concavity of each depression slide was placed a droplet of sterile sea water containing, per ml., 500 or 1000 units of penicillin G and 0.5 or 1 mg. of streptomycin. The lower concentrations were used in the experiments with *Uca* and the higher concentrations in the experiments with *Pagurus*. A crab selected to serve as a source of infected gill material was immersed briefly in 70% ethanol, rinsed in sterile sea water, and blotted on sterile filter paper. The legs were cut off close to the body with sterile scissors and the blood allowed to exude into the droplet of sea water with antibiotics. Two such blood-sea water mixtures could be prepared from one crab. In the experiments with *Uca* the blood was diluted by the sea water about 1 : 1, in those with *Pagurus* about 1 : 3. The carapace of the crab was then torn off. At this step, if the animal was close to molting, the old carapace cuticle would readily come loose revealing the soft newly formed skin beneath it. After exposure of the gill chamber, the gills were plucked off and placed in the mixtures of blood and sea water with antibiotics.

The preparations were kept at room temperatures in dim light and observed with a dissecting microscope for the appearance of trophonts. The gills were later placed between slide and coverslip and examined for the presence of phoronts and for possible signs of excystation.

Results

A. *Observations*

1. *Gymnodinioides* sp. of *Uca pugnax*. About 80% of the crabs of this species examined showed a few to many phoronts on their gills. The incidence of trophonts was, however, much lower. Out of a series of 105 recently shed skins only 24 contained trophonts. This might have been the result of very rapid engorgement and early escape of the trophonts from the exuviae, especially since molting usually occurred during the night. On the other hand, it might be that *Uca* is a relatively unfavorable host. The phoronts were frequently surrounded by a cellular reaction, often containing considerable brown pigment. Such a host reaction was never seen in *Pagurus*.

The living trophonts of this ciliate had a more pointed and twisted posterior end than those of *Gymnodinioides inkystans* from *Pagurus*. A silver preparation suggested 10 or 11 rather than 9 ciliary bands. The developmental cycle was typical of the genus *Gymnodinioides* (Chatton and Lwoff, 1935). More detailed morphological study would be needed for the precise identification of this organism, which does not quite fit any of the described species of *Gymnodinioides*.

In gill cuticle material taken from crabs found in the act of molting, small trophonts which had already excysted were present, as well as cysts showing a motile trophont within, and also seemingly unchanged resting phoronts. Cysts containing a motile trophont did not show the conspicuous large refractile granules present in most of the other phoronts. This might indicate a utilization of reserve food granules during the encysted state, a suggestion already made by Miyashita

(1933). The following observation on re-infection, however, is not entirely in accordance with this idea.

One *Uca* isolated in a small dish with a little sea water molted during the night. In the morning engorged trophonts were found swimming about in the dish. The crab was removed to a separate dish. By the following day cysts undergoing tomite formation were observed in the first dish and the crab was returned to it. The next day active tomites were noted in the water of this dish. One day later (three days after the molt) the crab was killed and its gills examined. Eighteen phoronts were found. All of these had few reserve granules and most had a large vacuole near the posterior end. In several this vacuole was seen to collapse and re-form slowly. Had these cysts not been found on a host which was known to have just molted and just been re-infected, they might have been considered "ripe" phoronts ready to excyst.

2. *Gymnodinioides inkystans* of *Pagurus longicarpus*. At least 80% of the molt skins of *Pagurus* examined during three summers contained moderate to large numbers of trophonts which were clearly *G. inkystans*. Phoronts were found on the plicae of the gills of most of the hermit crabs examined.

B. *Experiments in vitro*

1. *Gymnodinioides* sp. of *U. pugnax*. In an initial experiment gills from a crab in the act of molting were placed in a droplet of sea water and in sea water containing a segment of leg integument. Active small trophonts were already present in the gills, but nowhere else, at the time of the preparation. Eight hours later only small trophonts were seen in the preparation with sea water alone, but in the other preparation numerous partially to fully engorged trophonts were swimming about. A few were already encysting. By the second day many active tomites had been formed. Thus, once excystation had occurred, the engorgement and subsequent development *in vitro* were essentially normal.

In all the later experiments, with both host-parasite combinations, the methods detailed in the section on Materials and Methods were used. The general plan was to pair a crab judged to be not near the molt with one considered close to molting. The gills of each were divided into two portions. One portion was placed in a droplet of sea water with antibiotics plus blood of the same crab, and the second portion in a similar droplet with blood of the other crab.

Out of five experiments of this type in which numerous phoronts were present on the gills of both crabs, fully formed trophonts ready to excyst and showing slight movements were found in three. In these three experiments the crab judged to be near molting had milky blood, whereas in the other two experiments the crab considered near molting had clear or faintly turbid blood. In two of the three positive experiments the signs of excystation occurred only on gills of the crab near molting in its own blood. In the third, they occurred on gills of the crab near molting in the blood of the non-molting one. In no case did phoronts from the gills of the non-molting crab show signs of excysting.

2. *Gymnodinioides inkystans* of *P. longicarpus*. Seven experiments of the paired type were done in which phoronts were present on the gills of both the non-molting crab and the crab near molting. The following observations were made.

Exp. 1. Seven hours after placing of the gills in the blood-sea water-antibiotics mixture, four large trophonts were seen in the preparations containing gills from the crab near molting in its own blood, and one small trophont in the preparation of gills from this same crab in the blood of the non-molting crab.

Exp. 2. No trophonts were seen after seven hours, but after one day one large trophont was present in the preparation of gills from the crab near molting in its own blood, and two large trophonts in the preparation of gills from the crab near molting in the blood of the non-molting crab.

When the gills of the non-molting crab in its own blood were then examined under higher magnification, the unexpected observation was made of three phoronts which showed ciliary movement within the cyst (out of a total of 16 seen). Of 20 phoronts seen on the gills of the non-molting crab in blood of the one near molting, none showed movement although three had a large vacuole near the posterior end.

Exp. 3. A large trophont was present after one day in the gills from the crab near molting held with its own blood. None of the other preparations showed any indication of excystation.

Exp. 4. After one day four trophonts had developed from the gills of the crab near molting held with its own blood, and one from the gills of this crab with blood of the non-molting crab.

Exp. 5. Within seven hours six trophonts had developed from the gills of the crab near molting in its own blood, and one trophont from the gills of this crab in blood of the non-molting one. By the next day two additional trophonts had appeared in the latter preparation.

Exp. 6. Six trophonts developed within seven hours from the gills of the crab near molting in blood of the non-molting one. Partial drying had occurred in the preparation containing gills of the crab near molting in its own blood.

Exp. 7. No trophonts or signs of excystation could be found in any of the preparations.

Discussion

Excystation of both species of *Gymnodinioides* can evidently take place *in vitro* from phoronts which have not yet begun to excyst *in vivo*. The host crustacean must, however, be very close to molting time for this to occur, so that the statement of Chatton and Lwoff (1935) that these phoronts excyst only in response to molting of their host still holds. Phoronts from the gills of crabs near molting excysted, on the whole, a little better in the presence of the homologous blood than in the presence of blood from a non-molting crab. Miyashita (1933) noted that certain "ripe" cysts of *G. caridinae* from a fresh-water shrimp excysted within a few minutes after being placed under a coverslip in body fluid of the host, whereas many "unripe" cysts did not. It might be that the successful excystation occurred only with cysts which happened to have been taken from a shrimp near molting.

The results of the experiments described in the present paper indicate that the encysted phoronts of *Gymnodinioides* may require a series of stimuli from the host in order to prepare them for the final stimulus which produces excystation just before the actual molt. Such a course of events would be somewhat analogous to that described by Cleveland and Nutting (1955) for the sexual phenomena of the protozoa of *Cryptocercus*. Protozoa transferred from a roach at one stage of the

molting cycle to another at a different stage invariably died, whereas those transferred to another roach at the same stage continued their development.

Summary

Observations and experiments have been made with the encysted phoronts of *Gymnodinioides inkystans* on the gills of the hermit crab (*Pagurus longicarpus*) and of *Gymnodinioides* sp. on the gills of the fiddler crab (*Uca pugnax*). The phoronts of both species would excyst *in vitro*, in a mixture of crab blood with sea water and antibiotics, and give rise to engorged trophonts, only if the cysts were taken from gills of a crab which was near molting. This excystation occurred somewhat more readily in the presence of blood from the same crab, near the molt, than in the presence of blood from a crab not close to molting. It is concluded that the encysted phoronts probably require a series of stimuli from the host in order to prepare them for the final stimulus which produces excystation just before the actual molt.

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